J. Biochem. 130, 823–831 (2001)

Characterization of Phagosomal Subpopulations along Endocytic Routes in Osteoclasts and Macrophages[1]

Eiko Sakai,* Hisatsugu Miyamoto,† Kuniaki Okamoto,* Yuzo Kato,* Kenji Yamamoto,‡,2 and Hideaki Sakai*

*Departments of *Pharmacology and †Orthodontics, Nagasaki University School of Dentistry, Nagasaki 852-8588; and ‡Department of Pharmacology, Kyushu University Graduate School of Dental Science, Fukuoka 812-8582*

Received September 6, 2001; accepted October 4, 2001

Modifications occurring during the transformation of phagosomes into mature phagolysosomes were investigated in osteoclast-like cells (OCLs) and macrophages using latex beads as markers for the isolation of phagosomal compartments (LBC) at different time points after phagocytosis. In OCLs, newly formed LBC acquired cathepsin K, tartarate-resistant phosphatase (TRAP), lysosome-associated membrane protein-1 (Lamp-1), and cathepsin D, and rapidly lost annexin II in a time-dependent manner. The levels of Rab7 and c-Src in OCLs initially increased and then gradually decreased during the transformation from early to late endosomal LBC or phagolysosomes. Receptor activator of NF-κB (RANKL) significantly increased the LBC levels of cathepsin K, TRAP, and c-Src, whereas calcitonin decreased the LBC levels of cathepsin K, TRAP, and Rab7, indicating that the transformation of early to late endosomal elements and lysosomes in OCLs is also regulated by osteoclastogenesis regulatory factors. On the other hand, changes in the LBC levels of Lamp-1, cathepsin D, and annexin II in macrophages were comparable to those in OCLs. However, contrary to osteoclastic LBC, Rab7 levels of macrophage LBC decreased in a time-dependent manner. Macrophage LBC were devoid of cathepsin K, TRAP, and c-Src in all transformation stages. These observations suggest that OCLs and macrophages have different phagosome maturation mechanisms that involve the specific and regulated acquisition of markers from endocytic organelles. The results also demonstrate that the use of LBC is a useful system in which to identify and characterize molecules involved in these different endocytic pathways.

Key words: endocytosis, macrophage, osteoclast, phagosome.

Phagocytosis is the process by which cells internalize large particulate materials destined to be degraded by an endosome/lysosome system. The more active phagocytic cells are mononuclear phagocytes (monocytes and macrophages) and polymorphonuclear leucocytes (neutrophils and eosinophils). Although this process is important in controlling infectious diseases and in the turnover of senescent cells, very little is known about the events occurring after internalization that lead to the transformation of newly formed phagosomes into mature phagolysosomes. The available evidence indicates that phagosomes successively fuse with different intracellular compartments including late endosomes and lysosomes and thereby acquire marker molecules from these endocytic organelles. The fusion events between early phagosomes and intracellular compartments may confer cell-specific properties on endosomal subpopulations through the acquisition of marker molecules from characteristic endocytic organelles. However, it is poorly understood whether endocytic pathways vary in different cell types.

Osteoclasts are polarized and multinucleated cells responsible for bone resorption. The most characteristic feature of actively resorbing osteoclasts is the presence of a ruffled border, a convoluted plasma membrane structure adjacent to the bone surface, and the clear zone. These cells dissolve both bone mineral and matrix proteins by active secretion of protons and proteinases through the ruffled membrane into the extracellular resorption lacunae (*1–6*). It is also known that bone degradation products are endocytosed from the ruffled border membrane and transported and secreted to the opposite extracellular space *via* the basolateral membrane (*7, 8*). This transcytotic pathway is important in bone remodeling but poorly understood. Previously, collagen fragment-containing intracellular vacuoles have been observed in actively resorbing osteoclasts colocalizing with cathepin K (*9*) and cathepsin E (*10*). Cathepsin K is a specific cysteine proteinase that is exclusively expressed in osteoclasts (*3, 4, 11, 12*), whereas cathepsin E is a specific aspartic proteinase that has been identified in limited sources such as the gastrointestinal tract and lymphoid tissues (*13*). Since both enzymes are confined exclusively to the ruffled border membrane and

[1] This work was supported in part by a Grant-in-Aid (09771541) from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-92-642-6337, Fax: +81-92-642-6342, E-mail: kyama@dent.kyushu-u.ac.jp

Abbreviations: Lamp, lysosome-associated membrane protein; LBC, latex bead-containing compartments; M-CSF, macrophage-colony stimulating factor; OCL, osteoclast-like cell; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RANKL, receptor activator of NF-κB ligand; SDS, sodium dodecyl sulfate; TRAP, tartarate-resistant acid phosphatase.

the eroded bone surface, it is most likely that they play a role in the extracellular degradation of bone matrix proteins. However, the co-localization of collagen fragments with these enzymes in some endocytic vacuoles also suggests their involvement in the intracellular degradation of bone matrix proteins in the course of transcytosis.

Macrophages are also highly endocytic cells that are capable of internalizing a broad spectrum of both soluble and particulate molecules. It is now known that the phagocytotic system in macrophages is involved in immune processes, in addition to the inactivation of bacterial endotoxins and the metabolism of lipids, steroids, and proteins. Although much progress has been made in elucidating the properties of phagocytosis in macrophages, little is known about the regulation of the transformation of early phagosomes to mature phagolysosomes.

In the present study, we have attempted to characterize phagosomal subpopulations isolated from both osteoclasts and macrophages by the latex bead method. This method is a useful technique for qualitative and quantitative biochemical analyses of bead-associated proteins at different stages of phagosome formation and maturation (*14–16*). The results show that latex bead–containing compartments (LBC) in both cell types gradually transform with time and transiently display a protein composition characteristic of each cell type.

MATERIALS AND METHODS

Antibodies and Chemicals—Specific antibodies against cathepsin B (*17*), cathepsin D (*18*), and the C-terminal peptide of cathepsin K (*19*) were prepared as described previously. Antiserum against TRAP was a kind gift from Dr. G. Andersson (Division of Immunology Microbiology, Pathology, and Infectious Diseases Karolinska Institute, University Hospital, Sweden). Specific antibodies against Rab7 and Rab5 were kind gifts from Dr. E. Kominami (Department of Biochemistry, Juntendo University, Tokyo). Antibodies against Lamp-1 was a kind gift from Dr. K. Akasaki (Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Hiroshima). Antibodies against Rab3, c-Src, and annexin II were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human RANKL (sRANKL) was purchased from Pepro Tech EC LTD (London, UK). Eel calcitonin (elcatonin) was a kind gift from Asahi Chemical Industry (Tokyo). All chemicals used were of analytical grade.

Culture of Osteoclast-Like Cells (OCLs) and J774 Mouse Macrophages—Bone marrow cells were obtained from the tibiae and femora of 6–8-week-old male ddY mice. Bone marrow cells (5×10^6 cells) were co-cultured with primary-cultured osteoblasts (1×10^6 cells) prepared from the calvariae of newborn ddY mice in α-minimal essential medium (αMEM) containing 10% fetal bovine serum (FBS), 1α,25-dihydroxyvitamin D3 [$1\alpha,25(OH)_2D_3$] (10^{-8} M, kindly provided from Teijin, Tokyo), and prostaglandin E2 (10^{-6} M) in 100-mm-diameter dishes coated with collagen gel (Nitta Gelatin, Osaka) as described previously (*20*). After 7 days of culture, the cells were isolated from the gels by treatment with 0.1% collagenase (type 3, Worthington Biochemical, NJ, USA) and replated on plastic dishes for 2 h at 37°C in a 5% CO_2 atmosphere. Weakly adherent bone marrow and stroma cells were removed by treatment with 0.001% pronase E (Calbiochem, La Jolla, CA, USA) in 0.02% EDTA. Further incubation was carried out in the presence of 20 ng/ml macrophage-colony stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN, USA). More than 80% of the final adherent cells were TRAP-positive multinucleated cells and mononuclear cells. J774 mouse macrophages were obtained from Riken Cell Bank (Tokyo) and grown in 100 mm dishes in Dulbecco's modified Eagle's medium containing 10% FBS, and 1% glutamine (*21*).

Phagosome Formation and Cell Fractionation—The formation and isolation of phagosomes were carried out by the method of Desjardins *et al.* (*15, 16*) as described previously, with slight modifications. Phagosomes in OCLs and macrophages were formed by the internalization of latex beads (1.7 μm diameter, 2.5% suspension, yellow, Fluoresbrite TM, Polysciences, PA, USA) diluted 1:50 in culture medium at 37°C for 60 min. The cells were then washed in phosphate-buffered saline (PBS) (3 × 10 min) on ice, followed by a chase period of up to 24 h in medium without beads at 37°C. The cells were disrupted in homogenization buffer [3 mM imidazole, pH 7.4, containing 8.55% (w/w) sucrose, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml chymostatin, 1 μg/ml E-64, 1 μg/ml leupeptin, 1 μg/ml pepstatin] by nitrogen cavitation for 20 min at 300 psi in a bomb (Parr Instrument, Moline, IL) at 4°C. After centrifugation at 1,500 rpm for 7 min to remove nuclei and unbroken cells, the supernatants containing the latex bead–containing phagosomal compartments (LBC) were subjected to stepwise sucrose gradient centrifugation. The LBC supernatant was adjusted to 40% sucrose by adding 62% sucrose solution, and then loaded on a 1-ml cushion of 62% sucrose. Then, 2 ml of 35% sucrose, 2 ml of 25% sucrose, and 2 ml of 10% sucrose solution (pH 7.4) were layered. Centrifugation was carried out at 24,000 rpm for 1 h in a SW40 Hitachi swinging rotor. The LBC fractions were collected from the 10/25% sucrose interface. The collected LBC fractions were washed with PBS. The purity of the final LBC fractions was evaluated by electron microscopy.

Electron Microscopy—Cultured OCLs and macrophages were fixed with 2% paraformaldehyde, 1% glutaraldehyde, and 0.025% $CaCl_2$ in 0.05 M cacodylate buffer (pH 7.4). Post fixation was carried out in 1% OsO_4 in 0.05 M cacodylate buffer (pH 7.4), for 2 h, followed by dehydration in ethanol, and embedded in Quetol 812 resin. Ultra-thin sections were cut with a diamond knife and examined using a Hitachi H-800 electron microscope.

Western Blot Analysis—The kinetic association of various molecules in endocytic organelles with LBC was determined by Western immunoblotting and densitometric scanning analysis. The same number of LBC, as determined by the fluorescence intensity of the latex beads at 480 nm (excitation at 380 nm), was used in each experiment. At each time point, the LBC fractions from both cell types were dissolved in SDS buffer (2% SDS/100 mM dithiothreitol/10% glycerol/0.0025% bromophenol blue in 62.5 mM Tris-HCl, pH 6.8), and then subjected to SDS-PAGE under reducing conditions. Proteins separated in the gels were transferred onto polyvinylidene difluoride membranes. The antigenic sites were detected with an enhanced ECL kit (Amersham, Buckinghamshire, UK).

RESULTS

Microscopic View of Phagocytosing OCLs—OCLs derived from co-cultures of primary osteoblasts and bone marrow cells were plated on plastic dishes for 2 h at 37°C. Weakly adherent bone marrow and stroma cells were removed by treatment with pronase E and EDTA. More than 80% of the cells in this preparation were positive for TRAP, a marker for osteoclasts (Fig. 1A). The isolated OCLs were immunostained with specific antibodies against cathepsins K, D, and B (Fig. 2). Preferential immunoreactivity for cathepsin K was observed at the cell surface. Cathepsin K was also detected in some endocytic vacuoles in these cells. Cathepsin D immunoreactivity in the cells was found in vesicles and vacuoles distributed throughout the cytoplasm. This antigen was not detected on the cell surface of OCLs. Conversely, immunoreactivity for cathepsin B was barely detectable in OCLs. The immunohistochemical localization of these enzymes in osteoclasts was compared with that in macrophages (J774). Macrophages were devoid of the immunoreactivity for cathepsin K and activity staining for TRAP. However, the intracellular reactivity for cathepsins D and B was more intense than in OCLs. These two antigens were exclusively confined to the intracellular vesicles and vacuoles throughout the cytoplasm.

When the isolated OCLs were incubated with latex beads for 60 min at 37°C, most of the cells rapidly phagocytosed the latex beads (Fig. 3, A and B). Within a 4 h chase, newly formed LBC were localized in the center of the cytoplasm (Figs. 1B and 3, C and D). After a 24 h chase, LBC migrated to a more central area of the cytoplasm (Fig. 3, E and F). At the electron microscopic level, LBC were seen to be initially localized at or in the vicinity of the apical cell surface (Fig. 4A). After a 4 h of chase, LBC were surrounded by electron dense endosome-like ornagelles near

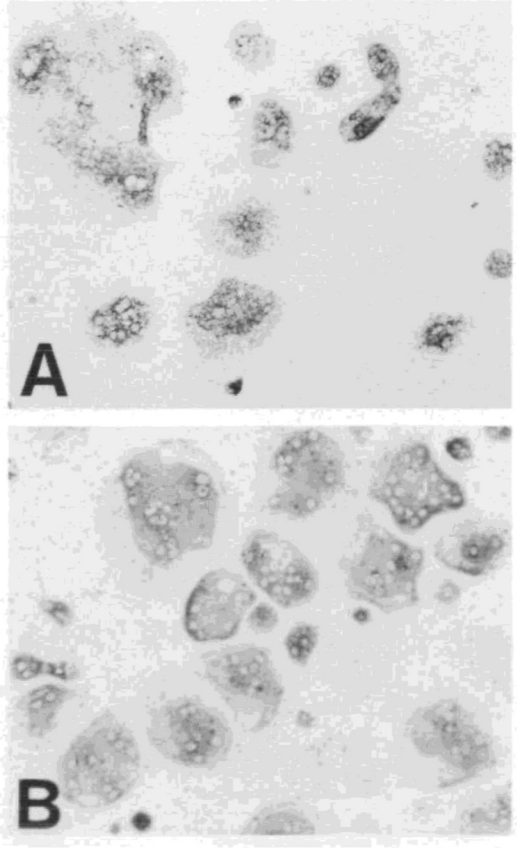

Fig. 1. **Morphological evaluation of the OCLs preparation from mouse bone marrow cells using activity staining for TRAP.** Mouse bone marrow cells were co-cultured with mouse primary osteoblasts in the presence of $1\alpha,25(OH)_2D_3$ (10^{-8} M) and PGE2 (10^{-6} M) for 7 days. After treatment with 0.1% collagenase, the detached cells were inoculated and cultured for 2 h on dishes. After removal of the less-adherent bone marrow and stroma cells with pronase E/EDTA, the remaining adherent cells were stained for TRAP and methyl-green (A). The OCLs fraction was incubated with latex beads for 1 h to allow phagocytosis to occur. After internalization, the cells were washed and chased for 4 h. Following fixation with 4% paraformaldehyde (PFA), the cells were stained for TRAP (B). Original magnification ×25.

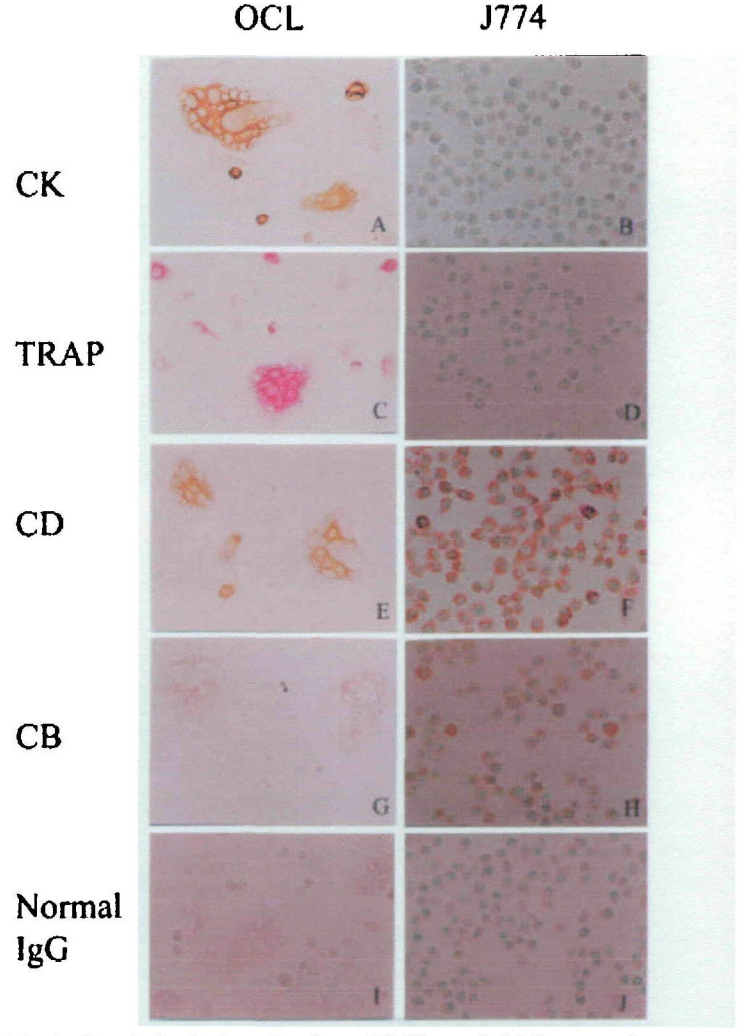

Fig. 2. **Morphological evaluation of OCLs and J774 macrophages, using various marker molecules for intracellular organelles.** Following fixation with 4% paraformaldehyde, both cell types were immunostained with antibodies specific for cathepsin K (A, B), cathepsin D (E, F), and cathepsin B (G, H) or normal rabbit IgG (I, J). Each cell type was also stained for TRAPase (C, D). Original magnification ×100. TRAP-positive OCLs contain the phagocytosed latex beads.

the ruffling membrane (Fig. 4B) and inside the cells (Fig. 4C).

Isolation of LBC from OCLs and Macrophages—After incubation with latex beads for 60 min at 37°C, OCLs and macrophages were washed and chased for various periods of time. After lysis by N_2 cavitation, the cells were subjected to step-wise sucrose density gradient centrifugation. As shown in Fig. 4D, the fraction obtained from OCLs at the 10/25% sucrose interface consisted of LBC surrounded by continuous and closely apposed membranes. No contamination by other organelles was observed in this fraction. The same results were obtained with macrophages (not shown).

Kinetic Analysis of the LBC Protein Compositions of OCLs and Macrophages—The protein compositions of LBC isolated from OCLs and macrophages in the course of the endocytic transport pathway were analyzed by immunoblotting with antibodies against various marker proteins followed by densitometric scanning. The LBC level of cathepsin K in OCLs gradually increased and reached a peak at around 6 h after phagocytosis (Figs. 5 and 6A). The high

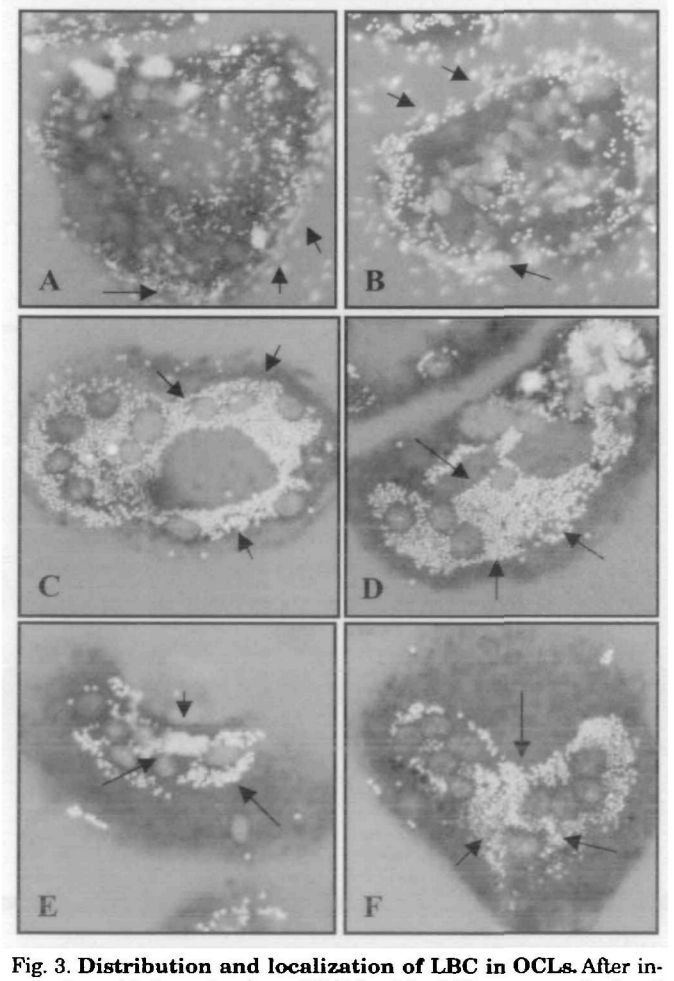

Fig. 3. **Distribution and localization of LBC in OCLs.** After incubation with latex beads at 37°C for 1 h, OCLs were washed and chased for various time periods. After fixation with 4% paraformaldehyde, the cells were stained for TRAPase and Hoechst33258. At a 0 h chase, latex beads were rapidly phagocytosed and LBC were preferentially localized on the cell surface of TRAP-positive multinucleated OCLs (A, B). After 4 h of chase, LBC were widely distributed throughout the cytoplasm of OCLs (C, D). At 24 h after phagocytosis, LBC migrated to a more central area of the cytoplasm of OCLs (E, F). Arrows indicate typical LBC localizations. Original magnification ×100.

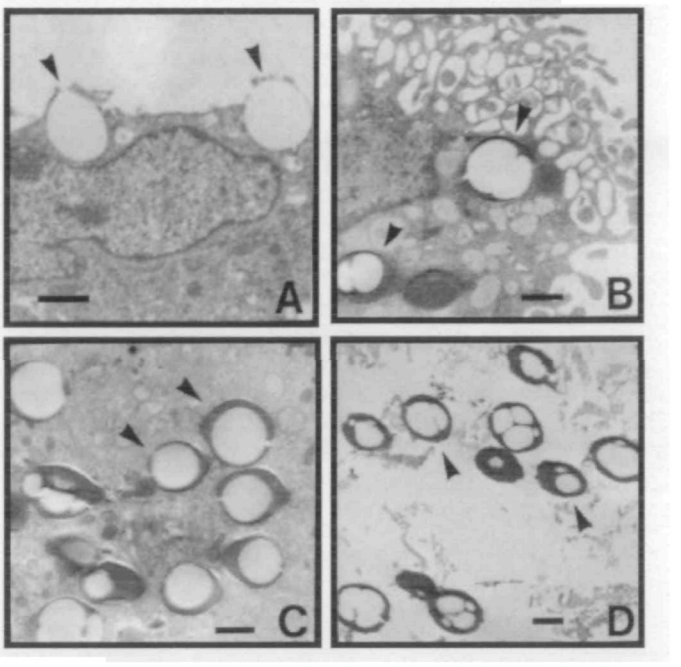

Fig. 4. **Electron micrographs showing LBC transfer from early to late endosomal elements or phagolysosomes.** After incubation with latex beads at 37°C for 1 h, OCLs were washed and chased for 0 (A) or 4 h (B, C). Immediately after phagocytosis, LBC were present at the cell surface of OCLs. At a 4 h chase, LBC were surrounded by electron-dense organelles and localized at or in the vicinity of the ruffling plasma membranes (B) and in the central area of the cytoplasm of OCLs (C). After lysis of the cells at 4 h after phagocytosis, the postnuclear supernatant was subjected to stepwise sucrose gradient centrifugation. The LBC fraction isolated at the interface of the 10–25% sucrose layers was analyzed by electron microscopy (D). Arrowheads indicate LBC. Bars indicate 1.0 μm.

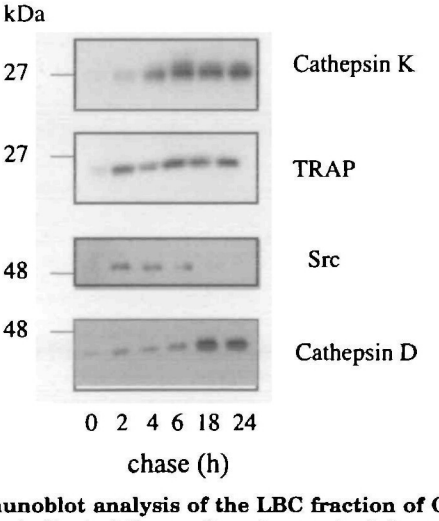

Fig. 5. **Immunoblot analysis of the LBC fraction of OCLs isolated at the indicated times after phagocytosis by antibodies specific for cathepsin K, TRAP, c-Src, and cathepsin D.** OCLs were incubated with latex beads for 1 h at 37°C and then chased without beads for the indicated times. The LBC fractions isolated by sucrose density gradient centrifugation were lysed and subjected to SDS-PAGE and immunoblotting with various antibodies.

level of LBC cathepsin K was maintained throughout the chase period up to 24 h. However, lysosomal cysteine proteinase cathepsin B was barely detectable in osteoclastic LBC throughout the chase period (Fig. 6A). In contrast, while the LBC cathepsin B level in macrophages increased rapidly and then decreased after phagocytosis, cathepsin K was not detected in the LBC throughout the chase period. The LBC levels of cathepsin D, a lysosomal aspartic proteinase, in both OCLs and macrophages increased in a time-dependent manner throughout the chase period, but the time-course to the peak values differed between the two cell types. The TRAP level in OCLs increased rapidly in the

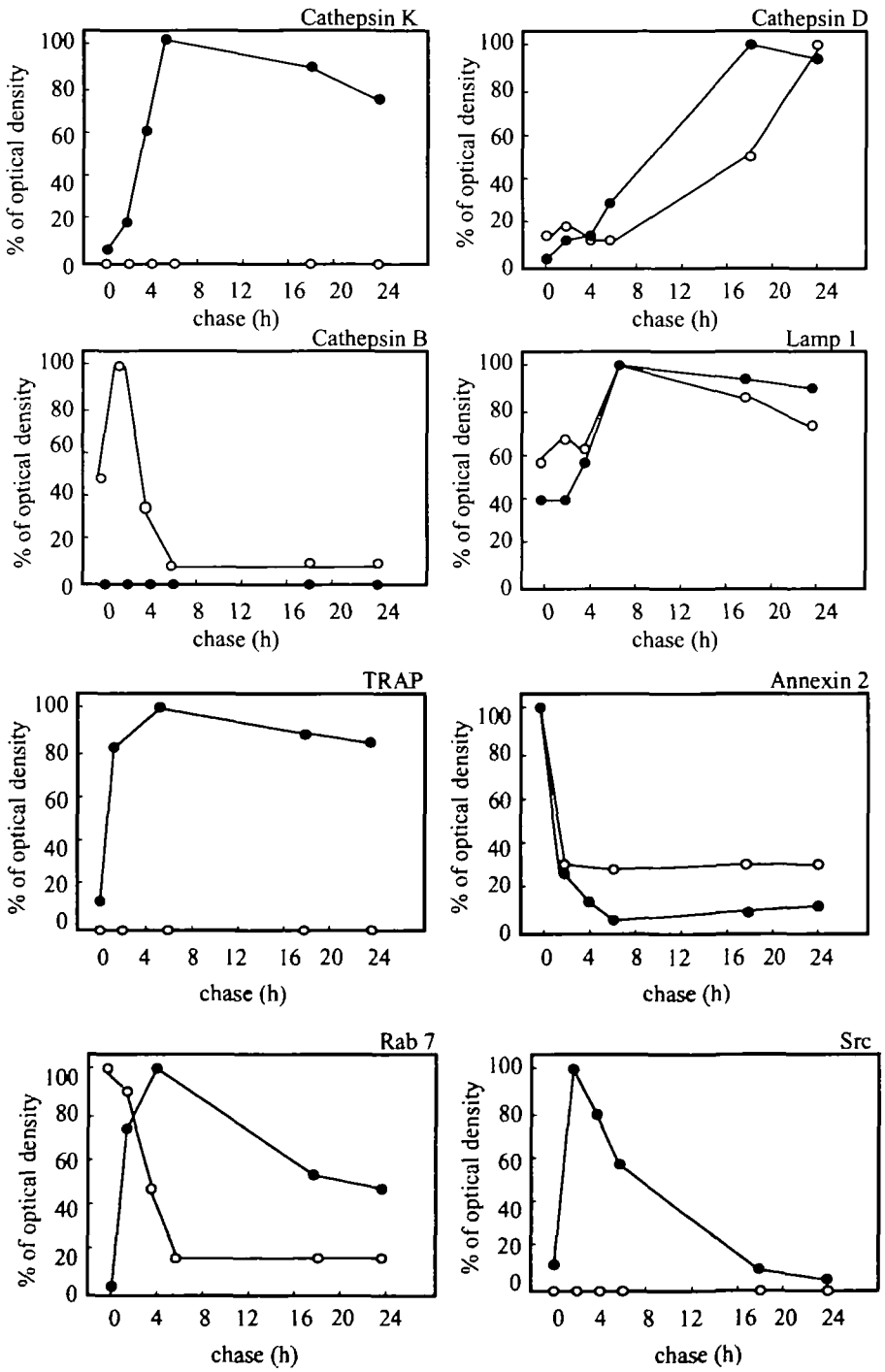

Fig. 6. **Changes in LBC levels of various marker proteins in OCLs and macrophages during the process of transformation from early LBC into late endosomal elements or phagolysosomes.** Both OCLs and macrophages were incubated with latex beads for 1 h at 37°C and then chased without beads for the indicated times. The LBC fractions isolated from both cell types were lysed and subjected to SDS-PAGE and immunoblotting with various antibodies. Protein levels were quantified by densitometric scanning. The open and closed circles indicate the data for macrophages and OCLs, respectively.

early LBC and reached a peak at around 2 h after phagocytosis. This high TRAP level in the LBC was maintained up to 24 h after phagocytosis. Conversely, TRAP was not detected in the macrophage LBC throughout the chase period (Fig. 6A).

Since c-Src tyrosine kinases are known to be essential for osteoclastic bone resorption (*22–26*) and to enhance phagocytosis in macrophages greatly (*27*), we next examined the c-Src level in LBC in OCLs and macrophages. The c-Src level in the osteoclastic LBC increased rapidly, reached a peak within 2 h after phagocytosis, and then decreased with time (Fig. 6B). The c-Src protein was no longer detectable in the late LBC after an 18 h chase. Interestingly, c-Src in the macrophage LBC was not detectable throughout the chase period (Fig. 6B).

Rab GTPases have been shown to be associated with distinct cellular compartments and to function as specific regulators of intracellular transport (for reviews see Ref. *28–31*). Therefore, Rab proteins have been used as compartment-specific markers as well as tools with which to characterize intracellular transport pathways. Among the members of this protein family, Rab7 has been shown to localize to late endosomes (*32*) and to regulate the transformation from early endosomes to late endosomes (*33, 34*) and lysosomes (*35*). On the other hand, Rab5 localizes to plasma membranes and early endosomes (*32, 36*) and has been shown to control transport from plasma membranes to sorting endosomes as well as the homotypic fusion of early endosomes (*36–38*). To gain further insight into the involvement of these small GTPases in the endocytic pathway, the LBC levels of Rab5 and Rab7 in OCLs and macrophages were examined. Rab7 was barely detectable in the osteoclastic LBC immediately after phagocytosis, but increased rapidly, reached a peak at around 4 h chase, and then gradually decreased up to a 24 h chase (Fig. 6B). Conversely, this protein accumulated greatly in the macrophage LBC during the 60-min incubation with latex beads. This accumulated Rab7 in the LBC was decreased exponentially during the chase period up to 6 h after phagocytosis. Neither the early endosomal marker protein Rab5 nor the secretory vesicle marker protein Rab3 was detectable in either osteoclastic or macrophage LBC throughout the chase period (not shown).

The lysosomal membrane-associated protein Lamp-1 is known to be directed to lysosomes after synthesis (*39, 40*). A minor part of Lamp-1 has been shown to be transported to lysosomes *via* the cell surface as an alternative transport pathway (*41–43*). The Lamp-1 level in the osteoclastic LBC increased time-dependently, reaching a plateau value at around 6 h after phagocytosis (Fig. 6A). This result is compatible with that obtained for the macrophage LBC. On the other hand, the Ca^{2+}-dependent phospholipid binding protein annexin II has been shown to be involved in the regulation of membrane trafficking events (for reviews see Ref. *44*). In addition to serving as a receptor for plasminogen, tissue-type plasminogen activator, and plasmin, annexin II is involved in direct vesicle transport to the apical cell surface of polarized MDCK cells (*45*). Therefore, we tested whether annexin II is involved in the transformation of early phagosomes to mature phagolysosomes in both cell types. Annexin II accumulated at high levels in the early LBC of both OCLs and macrophages during 60-min incubation with latex beads. The accumulated annexin II level

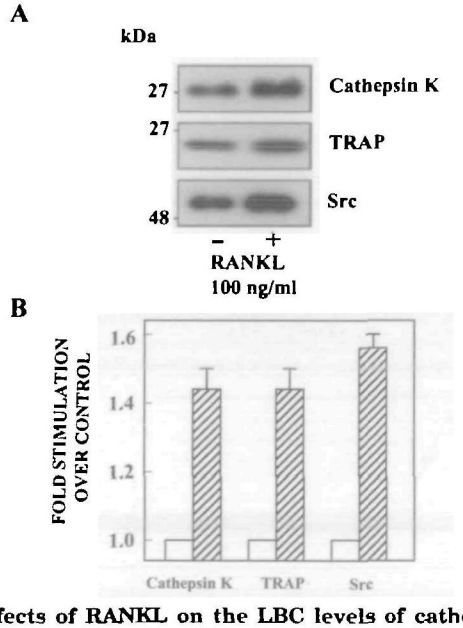

Fig. 7. **Effects of RANKL on the LBC levels of cathepsin K, TRAP, and c-Src in OCLs.** OCLs were incubated with latex beads for 1 h at 37°C in the presence of 20 ng/ml M-CSF and then chased without beads for 4 h in the presence or absence of 100 ng/ml RANKL. An equal number of LBC, as determined by the fluorescence intensity at 480 nm (excitation at 380 nm), were lysed and subjected to SDS-PAGE followed by immunoblotting with antibodies against each protein (A). The LBC levels of the respective proteins were determined using an imaging densitometric analyzer and expressed as relative ratios to the non-treated sample (B).

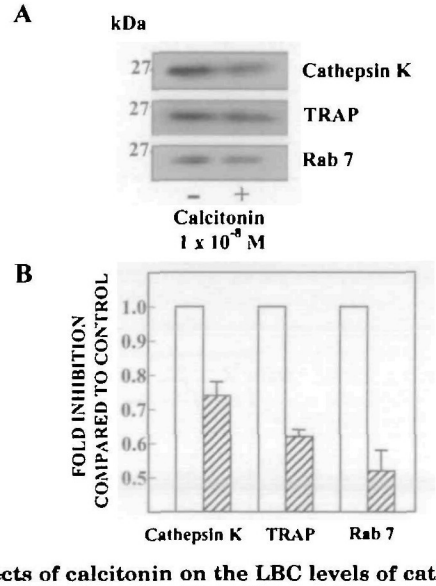

Fig. 8. **Effects of calcitonin on the LBC levels of cathepsin K, TRAP, and Rab7 in OCLs.** The cells were incubated with latex beads for 1 h at 37°C in the presence of 20 ng/ml M-CSF and then chased without beads for 4 h in the presence or absence of 10^{-8} M calcitonin. An equal number of LBC, as determined by the fluorescence intensity at 480 nm (excitation at 380 nm), were lysed and subjected to SDS-PAGE followed by immunoblotting with antibodies against each protein (A). The LBC levels of the respective proteins were determined using an imaging densitometric analyzer and expressed as relative ratios to the non-treated sample (B).

decreased with time throughout the chase period (Fig. 6B).

Effects of RANKL and Calcitonin on the Accumulation of Various Molecules in Osteoclastic LBC—The formation and activation of OCLs are now known to be regulated by various factors that are systemic, such as $1\alpha,25(OH)_2D_3$, calcitonin, and parathyroid hormone, as well as locally produced, such as IL-6 and RANKL, by osteoblasts, stroma cells, and immune cells (for reviews see Ref. *46*). To characterize the osteoclastic LBC further, OCLs were incubated with latex beads for 60 min at 37°C in the presence of M-CSF (20 ng/ml). After phagocytosis, the cells were treated with RANKL (100 ng/ml) or calcitonin (10^{-8} M) for 4 h at 37°C in the presence of M-CSF (20 ng/ml). The LBC levels of cathepsin K, TRAP, and c-Src were significantly increased (1.44 ± 0.09, 1.45 ± 0.08, and 1.55 ± 0.05-folds, respectively) (Fig. 7). The data suggest the stimulation of the transformation of newly formed phagosomes into late endosomes and phagolysosomes. In contrast, the treatment of OCLs with calcitonin significantly decreased the LBC levels of cathepsin K, TRAP, and Rab7 (0.74 ± 0.04, 0.62 ± 0.01, and 0.51 ± 0.08-fold, respectively) (Fig. 8), suggesting the suppression of the transformation of early endosomes into mature phagolysosomes. Neither RANKL nor calcitonin caused significant changes in the LBC levels of the tested proteins (not shown).

DISCUSSION

In this paper we describe studies on the endocytic flow of newly formed phagosomes and their fusion with intracellular organelles in mouse OCLs and macrophages using the latex bead method. It is well known that latex bead-loading phagosomes serve as useful markers for tracing their endocytic traffic and characterizing fusion events with intracellular organelles by which LBC acquire specific molecules. With this approach, phagosomal subpopulations at various stages of maturation were isolated and functionally characterized: early endosomal compartments, putative carrier vesicles, late endosomes, and phagolysosomes. In addition, as the exocytic vesicle marker Rab3 has not been detected in LBC of either cell type throughout the chase period, the results with this system indicate the characteristics of the endocytic transport pathways only.

After 1 h internalization at 37°C, latex beads were phagocytosed into the early endosomal elements of OCLs found in the cell periphery (Figs. 3 and 4). At 4 h after phagocytosis, the newly formed LBC were found in the perinuclear region. Considering that the late endosomal and lysosomal marker Lamp-1 and the lysosomal marker cathepsin D in the LBC showed maximal concentrations at 6 and 18 h after phagocytosis, respectively (Fig. 6), the LBC fractions between 4 and 6 h mainly consist of late endosomal elements, whereas after a 18 h chase, they represent phagolysosomal elements. Similar biochemical observations were obtained with macrophages.

It is clear from the present study that there are significant differences between the two cell types in the distribution and behavior of LBC proteins acquired by fusion with various intracellular organelles during the transformation into mature phagolysosomes. The acquisition pattern of Rab7 is strikingly different between OCLs and macrophages. The amount of Rab7 in osteoclastic LBC is very low immediately after phagocytosis but increases greatly within a 6 h chase and then gradually decreases. Its disappearance may represent the transformation of late endosomal LBC into phagolysosomes. Similar distribution profiles for Rab7 and Lamp-1 strongly suggest that Rab7 plays an important role in the transformation from early to late endosomal LBC or phagolysosomes. The results are compatible with previous observations that Rab7 localizes to late endosomes where it mediates transport from early to late endosomes (*33, 34*) or lysosomes (*35*). In contrast, Rab7 in the macrophage LBC accumulates maximally within 1 h of incubation with latex beads at 37°C, and rapidly decreases during the 6 h chase period, suggesting not only its rapid and efficient association with the late endosomal LBC during the first 1 h of incubation, but also the rapid transformation into phagolysosomes. Similarly, changes in the level of cathepsin B in the macrophage LBC shows the rapid transformation of early to late endosomal elements or phagolysosomes. Our results suggest that the transformation of early to late endosomes or lysosomes in macrophages is more rapid than in OCLs under the conditions used. On the other hand, Rab5 was not detected in LBC in either OCLs or macrophages throughout the chase period. However, it was found in early LBC formed after incubation with latex beads for 20 min at 37°C (not shown). Since Rab5 has been shown to localize to plasma membranes and early endosomes and is thought to control transport from plasma membranes to sorting endosomes or the fusion of early endosomes, the isolated LBC, particularly from macrophages, after 1 h internalization appears to be largely transformed from early to late endosomal LBC.

It has been shown that c-Src plays a critical role in bone resorption, and that deletion of the c-Src gene in mice results in defective osteoclasts and osteopetrosis (*25, 26*). However, the signaling pathway regulated by Src in the endocytic pathways in osteoclasts has not been identified. Recent experiments using macrophages also showed that Src protein kinases greatly enhance phagocytosis but are not absolutely essential for the process (*27*). Our results clearly show the localization of the c-Src protein in osteoclastic LBC and its association with the transformation of early into late endosomal elements or phagolysosomes. However, it is unlikely that c-Src in macrophages is involved in this process.

In addition to c-Src, the macrophage LBC does not contain cathepsin K or TRAP. Cathepsin K has been shown to be confined exclusively to the ruffled border membrane of osteoclasts and the eroded bone surface, suggesting its role in the extracellular degradation of bone matrix proteins (*3, 4, 11, 47*). The importance of cathepsin K in osteoclastic bone resorption is further supported by observations that patients with pycnodysostosis have mutations in their cathepsin K genes (*48*), and that osteoclasts from cathepsin K–deficient mutant mice cannot degrade bone matrix proteins (*49*). Our results show that cathepsin K accumulates with time in the LBC of OCLs, and that its level is maintained after a 24 h chase, indicating that this enzyme is transported to the late endosomal LBC and phagolysosomes where it appears to participate in the intracellular degradation of the endocytosed matrix proteins. The acquisition profile of TRAP in the LBC is similar to that of cathepsin K. Since TRAP has been shown to localize to transcytotic vesicles in osteoclasts and to generate reactive oxygen species, and thereby, perhaps be able to destroy

endocytosed proteins (*50*). Together, our present results suggest that TRAP contributes to the endosomal/lysosomal degradation of bone matrix proteins.

Furthermore, our results indicate that osteoclastogenesis regulating factors, such as RANKL and calcitonin, also regulate the association of cathepsin K, TRAP, c-Src, and Rab7 (Figs. 7 and 8). RANKL has recently been identified as an osteoclast differentiation factor (*51–54*), and is known to promote osteoclast bone resorption activity by inducing actin ring formation (*55*). Calcitonin is well known to induce a marked decrease in bone resorption by its receptor-mediated signaling pathway (*56*). The LBC levels of cathepsin K, TRAP, and c-Src in OCLs 4 h after phagocytosis are significantly enhanced by treatment with RANKL. In contrast, calcitonin greatly decreases the association of cathepsin K, TRAP, and Rab7 with the LBC. It is evident from the present study that the transformation of early into late endosomal LBC or phagolysosomes is also regulated by osteoclastogenesis regulating factors.

We thank Drs. M. Shibata and K. Kanaoka for fruitful discussions and Ms. M. Okaji and T. Iwamoto for technical assistance.

REFERENCES

1. Baron, R., Neff, L., Louvard, D., and Courtoy, P.J. (1985) Cell-mediated extracellular acidification and bone resorption: Evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. *J. Cell Biol.* **101**, 2210–2222
2. Blair, H.C., Teitelbaum, S.L., Ghiselli, R., and Gluck, S. (1989) Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science* **245**, 855–857
3. Drake, F.H., Dodds, R.A., James, I.E., Connor, J.R., Debouck, C., Richardson, S., Lee-Rykaczewski, E., Coleman, L., Rieman, D., Barthlow, R., Hastings, G., and Gowen, M. (1996) Cathepsin K, but not cathepsin B, L, or S, is abundantly expressed in human osteoclasts. *J. Biol. Chem.* **271**, 12511–12516
4. Tezuka, K., Tezuka, Y., Maejima, A., Sato, T., Nemoto, K., Kamioka, H., Hakeda, Y., and Kumegawa, M. (1994) Molecular cloning of a possible cysteine proteinase predominantly expressed in osteoclasts. *J. Biol. Chem.* **269**, 1106–1109
5. Vaananen, H.K., Karhukorpi, E.K., Sundquist, K., Wallmark, B., Roininen, I., Hentunen, T., Tuukkanen, J., and Lakkakorpi, P. (1990) Evidence for the presence of a proton pump of the vacuolar H^+-ATPase type in the ruffled borders of osteoclasts. *J. Cell Biol.* **111**, 1305–1311
6. Wucherpfennig, A.L., Li, Y.P., Stetler-Stevenson, W.G., Rosenberg, A.E., and Stashenko, P. (1994) Expression of 92 kD type IV collagenase/gelatinase B in human osteoclasts. *J. Bone Miner. Res.* **9**, 549–556
7. Nesbitt, S.A. and Horton, M.A. (1997) Trafficking of matrix collagens through bone-resorbing osteoclasts. *Science* **276**, 266–269
8. Salo, J., Lehenkari, P., Mulari, M., Metsikko, K., and Vaananen, H.K. (1997) Removal of osteoclast bone resorption products by transcytosis. *Science* **276**, 270–273
9. Yamaza, T., Goto, T., Kamiya, T., Kobayashi, Y., Sakai, H., and Tanaka, T. (1998) Study of immunoelectron microscopic localization of cathepsin K in osteoclasts and other bone cells in the mouse femur. *Bone* **23**, 499–509
10. Yoshimine, Y., Tsukuba, T., Isobe, R., Sumi, M., Akamine, A., Maeda, K., and Yamamoto, K. (1995) Specific immunocytochemical localization of cathepsin E at the ruffled border membrane of active osteoclasts. *Cell Tissue Res.* **281**, 85–91
11. Bossard, M.J., Tomaszek, T.A., Thompson, S.K., Amegadzie, B.Y., Hanning, C.R., Jones, C., Kurdyla, J.T., McNulty, D.E., Drake, F.H., Gowen, M., and Levy, M.A. (1996) Proteolytic activity of human osteoclast cathepsin K. *J. Biol. Chem.* **271**, 12517–12524
12. Faust, J., Lacey, D., Hunt, P., Burgess, T.L., Scully, S., Van, G., Eli, A., Qian, Y., and Shalhoub, V. (1999) Osteoclast markers accumulate on cells developing from human peripheral blood mononuclear precursors. *J. Cell Biochem.* **72**, 67–80
13. Sakai, H., Saku, T., Kato, Y., and Yamamoto, K. (1989) Quantitation and immunohistochemical localization of cathepsins E and D in rat tissues and blood cells. *Biochim. Biophys. Acta* **991**, 367–375
14. Werb, Z. and Cohn, Z.A. (1972) Plasma membrane synthesis in the macrophage following phagocytosis of polystyrene latex particles. *J. Biol. Chem.* **247**, 2439–2446
15. Desjardins, M., Huber, L.A., Parton, R.G., and Griffiths, G. (1994) Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. *J. Cell Biol.* **124**, 677–688
16. Desjardins, M., Celis, J.E., van Meer, G., Dieplinger, H., Jahraus, A., Griffiths, G., and Huber, L.A. (1994) Molecular characterization of phagosomes. *J. Biol. Chem.* **269**, 32194–32200
17. Yamamoto, K., Kamata, O., Takeda, M., and Kato, Y. (1983) Characterization of rat spleen cathepsin B and different effects of anti-inflammatory drugs on its activity. *Jpn. J. Oral Biol.* **25**, 834–838
18. Yamamoto, K., Kamata, O., Katsuda, N., and Kato, K. (1980) Immunochemical differences between cathepsin D and cathepsin E-like enzyme from rat spleen. *J. Biochem.* **87**, 511–516
19. Kamiya, T., Kobayashi, Y., Kanaoka, K., Nakashima, T., Kato, Y., Mizuno, A., and Sakai, H. (1998) Fluorescence microscopic demonstration of cathepsin K activity as the major lysosomal cysteine proteinase in osteoclasts. *J. Biochem.* **123**, 752–759
20. Akatsu, T., Tamura, T., Takahashi, N., Udagawa, N., Tanaka, S., Sasaki, T., Yamaguchi, A., Nagata, N., and Suda, T. (1992) Preparation and characterization of a mouse osteoclast-like multinucleated cell population. *J. Bone Miner. Res.* **7**, 1297–1306
21. Burkhardt, J., Huber, L.A., Dieplinger, H., Blocker, A., Griffiths, G., and Desjardins, M. (1995) Gaining insight into a complex organelle, the phagosome, using two-dimensional gel electrophoresis. *Electrophoresis* **16**, 2249–2257
22. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) Targeted disruption of the c-*src* proto-oncogene leads to osteopetrosis in mice. *Cell* **64**, 693–702
23. Horne, W.C., Neff, L., Chatterjee, D., Lomri, A., Levy, J.B., and Baron, R. (1992) Osteoclasts express high levels of pp60 c-src in association with intracellular membranes. *J. Cell Biol.* **119**, 1003–1013
24. Tanaka, S., Takahashi, N., Udagawa, N., Sasaki, T., Fukui, Y., Kurokawa, T., and Suda, T. (1992) Osteoclasts express high levels of $p60^{c\text{-}src}$, preferentially on ruffled border membranes. *FEBS Lett.* **313**, 85–89
25. Lowe, C., Yoneda, T., Boyce, B.F., Chen, H., Mundy, G.R., and Soriano, P. (1993) Osteopetrosis in Src-deficient mice is due to an autonomous defect of osteoclasts. *Proc. Natl. Acad. Sci. USA* **90**, 4485–4489
26. Lowell, C.A. and Soriano, P. (1996) Knockouts of Src-family kinases: stiff bones, wimpy T cells, and bad memories. *Gene Dev.* **10**, 1845–1857
27. Fitzer-Attas, C.J., Lowry, M., Crowley, T., Finn, A.J., Meng, F., DeFranco, A.L., and Lowell, C.A. (1998) Fcγ receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases. *J. Exp. Med.* **191**, 669–682
28. Pfeffer, S.R. (1994) Rab GTPases: master regulators of membrane trafficking. *Curr. Opin. Cell Biol.* **6**, 522–526
29. Nuoffer, C. and Balch, W.E. (1994) GTPases: multifunctional molecular switches regulating vesicular traffic. *Annu. Rev. Biochem.* **63**, 949–990
30. Novick, P. and Zerial, M. (1997) The diversity of rab proteins in vesicle transport. *Curr. Opin. Cell Biol.* **9**, 496–504
31. Olkkonen, V.M. and Stenmark, H. (1997) Role of Rab GTPases in membrane traffic. *Int. Rev. Cytol.* **176**, 1–85
32. Chavrier, P., Parton, R.G., Hauri, H.P., Simons, K., and Zerial, M. (1990) Localization of low molecular weight GTP binding

proteins to exocytic and endocytic compartments. *Cell* **62**, 317–319
33. Feng, Y., Press, B., and Wandinger-Ness, A. (1995) Rab 7: an important regulator of late endocytic membrane traffic *J. Cell Biol.* **131**, 1435–1452
34. Vitelli, R., Santillo, M., Lattero, D., Chiariello, M., Bifulco, M., Bruni, C.B., and Bucci, C. (1997) Role of the small GTPase RAB7 in the late endocytic pathway. *J. Biol. Chem.* **272**, 4391–4397
35. Meresse, S., Gorvel, J.P., and Chavrier, P. (1995) The rab7 GTPase resides on a vesicular compartment connected to lysosomes. *J. Cell Sci.* **108**, 3349–3358
36. Bucci, C., Parton, R.G., Mather, I.H., Stunnenberg, H , Simons, K., Hoflack, B., and Zerial, M. (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell* **70**, 715–728
37. Gorvel, J.-P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991) Rab5 controls early endosome fusion in vitro. *Cell* **64**, 915–925
38. McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M., and Smythe, E. (1998) A novel role for rab5-GDI in ligand sequestration into clathrin-coated pits. *Curr. Biol.* **8**, 34–45
39. D'Souza, M.P. and August, J.T. (1986) A kinetic analysis of biosynthesis and localization of a lysosome-associated membrane glycoproteins. *Arch. Biochem. Biophys.* **249**, 522–532
40. Green, S.A., Zimmer, K.-P., Griffiths, G., and Mellman, I. (1987) Kinetics of intracellular transport and sorting of lysosomal membrane and plasma membrane proteins. *J. Cell Biol.* **105**, 1227–1240
41. Lippincott-Schwarz, J. and Fambrough, D.M. (1986) Lysosomal membrane dynamics: structure and interorganellar movement of a major lysosomal membrane glycoprotein. *J. Cell Biol.* **102**, 1593–1605
42. Furuno, K., Yano, K., Akasaki, K., Tanaka, Y., Yamaguchi, Y., Tsuji, H., Himeno, M., and Kato, K. (1989) Biochemical analysis of the movement of a major lysosomal membrane glycoprotein in the endocytic membrane system. *J. Biochem.* **106**, 717–722
43. Mane, S.M., Marzella, L., Bainton, D.F., Holt, V.K., Cha, T., Hildreth, K., and August, J.T. (1989) Purification and characterization of human lysosomal membrane glycoproteins. *Arch. Biochem. Biophys.* **268**, 360–378
44. Waisman, D.M. (1995) Annexin II tetramer: structure and function. *Mol. Cell Biochem.* **149/150**, 301–322
45. Fiedler, K., Kellner, R., and Simons, K. (1997) Mapping the protein composition of trans-Golgi network (TGN)-derived carrier vesicles from polarized MDCK cells. *Electrophoresis* **18**, 2613–2619
46. Reddy, S.V. and Roodman, G.D. (1998) Control of osteoclast differentiation. *Crit. Rev. Eukaryot. Gen Exp.* **8**, 1–17
47. Bromme, D., Okamoto, K., Wang, B.B., and Biroc, S. (1996) Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. *J. Biol. Chem.* **271**, 2126–2132
48. Gelb, B.D., Shi, G.P., Chapman, H.A., and Desnick, R.J. (1996) Pycnodysostosis, a lysosomal disease cause by cathepsin K deficiency. *Science* **273**, 1236–1238
49. Saftig, P., Hunziger, E., Wehmeyer, O., Jones, S., Boyde, A., Rommerskirch, W., Moritz, J., Schu, P., and von Figra, K. (1998) Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin K-deficient mice. *Proc. Natl. Acad. Sci. USA* **95**, 13453–13458
50. Halleen, J.M., Raisanen, S., Salo, J.J., Reddy, S.V., Roodman, G.D., Hentunen, T.A., Lehenkari, P.P., Kaija, H., Vihko, P., and Vaananen, H.K. (1999) Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase. *J. Biol. Chem.* **274**, 22907–22910
51. Anderson, D.M., Maraskovsky, E., Billingsley, W.L., Dougall, W.C., Tometsko, M.E., Roux, E.R., Teepe, M.C., DuBose, R.F., Cosman, D., and Galibert, L. (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390**, 175–179
52. Lacey, D.L., Timms, E., Tan, H.L., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C , Eli, A., Qian, Y.X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J., and Boyle, W.J. (1998) Osteoprotegrin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93**, 165–176
53. Wong, B.R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett III, F.S., Frankel, W.N., Lee, S Y., and Choi, Y. (1997) TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J. Biol. Chem.* **272**, 25190–25194
54. Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashino, K., Udagawa, N., Takahashi, N., and Suda, T. (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. USA* **95**, 3597–3602
55. Burgess, T.L., Qian, Y., Kaufman, S., Ring, B.D., Van, G., Capparelli, C., Kelley, M., Hsu, H., Boyle, W.J., Dunstan, C.R., Hu, S., and Lacey, D.L. (1999) The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J. Cell Biol.* **145**, 527–538
56. Baron, R., Neff, L., Brown, W., Louvard, D., and Courtoy, P.J. (1990) Selective internalization of the apical plasma membrane and rapid redistribution of lysosomal enzymes and mannose 6-phosphate receptors during osteoclast inactivation by calcitonin. *J. Cell Sci.* **97**, 439–447