Endocytic pathway from the basal plasma membrane to the ruffled border membrane in bone-resorbing osteoclasts

Harri Palokangas*, Mika Mulari and H. Kalervo Väänänen
Department of Anatomy and Biocenter, University of Oulu, Kajaanintie 52 A, FIN-90220 Oulu, Finland

*Author for correspondence (e-mail: htp@raita.oulu.fi)

SUMMARY

We have characterized the convoluted ruffled border (RB) membrane that an activated osteoclast maintains against the bone matrix. The bulk of both lgp110 and rab7, a small GTP-binding protein participating in vesicle fusion to late endosomes, was localized to the RB. This indicates that the membrane has some characteristics of late endosomal membranes in other cells. Furthermore, the bulk of membrane-bound rab7 on the RB suggests that endocytic membrane transport is oriented towards the RB in resorbing osteoclasts. Consistently, both luminal horseradish peroxidase and receptor-bound transferrin, a marker of the early endosomal recycling pathway, were efficiently endocytosed from the basal plasma membrane and delivered to the RB. Delivery of membrane-associated transferrin to the RB further indicates that the RB is compositionally different from lysosomes and suggests that the endocytic pathway contributes to the maintenance of functional RB. In addition to transporting receptor-bound cargo to the RB, the endocytic pathway could act in balancing the membrane traffic associated with transcytosis from the RB to the basal plasma membrane. Endocytic processes (retrieval of mannose 6-phosphate receptors) in osteoclasts appeared to be fairly sensitive to bafilomycin A1, a specific inhibitor of vacuolar-type proton ATPases. Thus blocking the endocytic membrane traffic towards the RB could explain the inactivation of cells by low concentrations of the drug.

Key words: Bafilomycin A1, Bone resorption, Endosome, Osteoclast, Väänänen, 1991), which makes interpretation of experiments difficult. By using immunocytochemistry, Baron et al. (1988) characterized the biosynthetic pathway delivering lysosomal enzymes to the resorption lacuna. Recently, using enveloped viruses as tools, the basal surface of a resorbing osteoclast was shown to be divided into two separate domains, which receive either basolaterally or apically targeted viral glycoproteins, respectively (Salo et al., 1996). Recent studies (Salo et al., 1997; Nesbitt and Horton, 1997) also suggest that degraded matrix components are removed from the lacuna via a transcytotic vesicle transport process oriented towards the centrally located “functional secretory domain” of the basal plasma membrane.

In the present study we have characterized the RB of osteoclasts in more detail using rat osteoclasts cultured in vitro on devitalized bovine bone slices. Our studies indicate that the RB is not compositionally identical to the membrane of secondary lysosomes in other cells. In analogy to the phagocytic tubulocisternal organelle in macrophages (Rabinowitz et al., 1992), the RB contains both lysosomal glycoprotein lgp110 and rab7, a marker of a late endosomal compartment. Endocytic tracers, both luminal (horseradish peroxidase) and membrane-bound (transferrin), endocytosed from the basal plasma membrane were delivered to the RB. This indicates that the endocytic pathway, re-oriented during osteoclast activation, participates in the maintenance of the RB and could allow membrane flow

INTRODUCTION

Osteoclasts are multinucleated cells specialized for bone matrix degradation. The resorption lacuna that forms beneath a recruited osteoclast is lined by an intensively convoluted membrane, the ruffled border (RB). Vacular-type proton ATPases are concentrated on this membrane and acidify the resorption lacuna, thereby causing the dissolution of inorganic minerals and degradation of the organic matrix (Baron et al., 1985, 1988; Blair et al., 1989; Väänänen et al., 1990). The osteoclast isolates the resorption lacuna from the extracellular milieu by forming a tight contact area, i.e. a sealing zone, with the bone matrix. Cytoskeletal proteins (actin, vinculin, talin) are highly concentrated at the sealing zone and arranged in a polarized, ring-like manner (Lakkakorpi et al., 1989, 1993). Because of proteolytic enzyme content, acidic luminal pH, and glycoproteins typical of lysosomal membranes on its lining membrane (Baron et al., 1985), the resorption lacuna has been generally considered to be a specialized ‘extracellular lysosome’.

The vesicle transport processes in a bone-resorbing osteoclast are poorly known. Two methodological problems in particular have made these studies difficult. First, obtaining cultures of pure and active osteoclasts in vitro has not been well-established. Secondly, the individual osteoclasts are at different stages of the resorption cycle (Lakkakorpi and Väänänen, 1991), which makes interpretation of experiments difficult. By using immunocytochemistry, Baron et al. (1988) characterized the biosynthetic pathway delivering lysosomal enzymes to the resorption lacuna. Recently, using enveloped viruses as tools, the basal surface of a resorbing osteoclast was shown to be divided into two separate domains, which receive either basolaterally or apically targeted viral glycoproteins, respectively (Salo et al., 1996). Recent studies (Salo et al., 1997; Nesbitt and Horton, 1997) also suggest that degraded matrix components are removed from the lacuna via a transcytotic vesicle transport process oriented towards the centrally located “functional secretory domain” of the basal plasma membrane.

In the present study we have characterized the RB of osteoclasts in more detail using rat osteoclasts cultured in vitro on devitalized bovine bone slices. Our studies indicate that the RB is not compositionally identical to the membrane of secondary lysosomes in other cells. In analogy to the phagocytic tubulocisternal organelle in macrophages (Rabinowitz et al., 1992), the RB contains both lysosomal glycoprotein lgp110 and rab7, a marker of a late endosomal compartment. Endocytic tracers, both luminal (horseradish peroxidase) and membrane-bound (transferrin), endocytosed from the basal plasma membrane were delivered to the RB. This indicates that the endocytic pathway, re-oriented during osteoclast activation, participates in the maintenance of the RB and could allow membrane flow
to the RB, compensating for the loss of membrane caused by the transcytotic secretory pathway. Endocytic processes in osteoclasts were affected by Bafilomycin A1 (Baf), a specific inhibitor of vacuolar proton ATPases (Bowman et al., 1988), at low concentrations. This result is in accordance with the reported pH sensitivity of endocytic processes in other cell types (Clague et al., 1994; Reaves and Banting, 1994; Chapman and Munro, 1994; van Weert et al., 1995; van Deurs et al., 1996), and could explain the sensitivity of osteoclast resorption activity to this drug (Sundquist et al., 1990).

MATERIALS AND METHODS

Isolation and culture of osteoclasts

Cell culture materials were obtained from Life Technologies, Inc. The procedure used to culture rat osteoclasts was modified from the original method developed by Boyle et al. (1984) and Chambers et al. (1984) and has been described earlier by Lakkakorpi et al. (1989). Osteoclasts were mechanically disaggregated from long bones of 1-3 day old rat pups in DME buffered with 20 mM Hepes and containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FCS, pH 6.9 (DME-Hepes-FCS). The disaggregated cells were attached to de-vitalized bovine cortical bone slices (100-150 μm thick) at 37°C in 5% CO₂/95% air for 30 minutes. The non-attached cells were then washed away and the remaining cells on bone slices were cultured for 48 hours in DME-Hepes-FCS and were then used for experiments. Baf was a kind gift from K. Altmendorf (Osnabrück, Germany).

Antibodies and immunofluorescence

Polyclonal antibodies recognizing lgp110 and monoclonal antibody to lgp120 were kindly provided by P. Luzio (Cambridge, UK). Polyclonal, affinity-purified anti-rab7 antibodies were provided by M. Zerial (EMBL, Heidelberg, Germany). The antisera against cation-independent and cation-dependent mannose 6-phosphate receptors (CI-MPR, CD-MPR) were gifts from V. Marjomäki (Jyväskylä, Finland) and K. von Figura (Göttingen, Germany), respectively. Both the monoclonal antibody to the extreme N terminus of rat TGN38 (2F7.1) and polyclonal antibodies to TGN38 were provided by G. Banting (Bristol, UK). Monoclonal antibody to mannosidase II was obtained from Berkeley Antibodies Co. (Richmond, CA, USA). VSV G protein was localized by using polyclonal antiserum (provided by K. Metsikko, Oulu, Finland). Iron-loaded transferrin (Fe-Tf) was localized using either polyclonal antibodies (Zymed, CA, USA) or a monoclonal antibody (BioGenex, CA, USA). Monoclonal antibody against rat transferrin receptor (TfR) was provided by W. Partridge (UCLA, Los Angeles, USA). Vitrinectin receptor (αβ3) was immunolocalized using monoclonal antibody (F11), a gift from M. Horton (London, UK). TRITC- and FITC-conjugated phalloidin (Sigma) were used to stain F-actin. Nuclei were stained with Hoechst 33258 (Sigma) at 1.3 μg/ml for 5 minutes.

Generally, for immunofluorescence studies cells were fixed with 3% paraformaldehyde in phosphate-buffered saline, pH 7.4, for 20 minutes. To immunolocalize rab7, cells were treated with 0.5% saponin in Na-Pipes buffer, pH 6.8, for 5 minutes prior to fixation, to release cytoplasmic free protein (Chavrier et al., 1990). For the localization of IgG110, IgG120, the MPRs and TfR, fixation of antigens with ice-cold methanol for 6 minutes was used.

The secondary antibodies used were TRITC-conjugated swine anti-rabbit IgG from Dako A/S (Glostrup, Denmark) and FITC-conjugated goat anti-mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania, USA). To enhance the immunofluorescence detection of Fe-Tf on the plasma membrane we used Texas Red-coupled goat anti-rabbit and donkey anti-goat secondary antibodies (Jackson ImmunoResearch Laboratories) successively. Peroxidase-conjugated goat anti-rabbit antibodies were from Dako A/S.

Samples were viewed with a Leitz fluorescence microscope or a confocal laser scanning microscope (CLSM) consisting of a Leitz fluorescence microscope and Leica Lasertecnhic GmbH 1.05 software (Heidelberg, Germany) equipped with a multiline 750 mW Omnichrome argon-cyanotron laser (Chino, CA, USA).

Experiments with endocytic tracers

Osteoclasts were incubated with horseradish peroxidase (HRP) from Sigma (10 mg/ml, type VI-A, P-6782) in DME supplemented with 20 mM Hepes, 0.84 g/l bicarbonate, 2 mM L-glutamine and 1% BSA, pH 6.9 (DME-Hepes-BSA) for 1 hour at 37°C. Cells were fixed with 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 30 minutes. DAB-reaction was then performed and the cells were further fixed with 2.5% glutaraldehyde in phosphate buffer for 2 hours and processed for electron microscopy (see Electron Microscopy).

Human transferrin (Sigma, T-2252) was iron loaded and FITC conjugated according to Klausner et al. (1983). Osteoclasts were incubated with iron-loaded transferrin (Fe-Tf) (0.1 mg/ml) in DME-Hepes-BSA and subsequently fixed with 3% paraformaldehyde in phosphate-buffered saline for 20 minutes. For immunoelectron-microscopic localization of Fe-Tf, the cells were fixed with McLean and Nakane’s peridate-lysine-paraformaldehyde (PLP) fixative for 2.5 hours. Staining using polyclonal anti-Tf antibodies and the immunoperoxidase reaction were carried out according to Brown and Farquhar (1989).

Experiments with Acidrine Orange

Osteoclasts were incubated with Acidrine Orange (5 μg/ml) (Merck, Germany) in DME-Hepes-FCS for 15 minutes at 37°C. After washing, the cells were mounted in warm medium and studied immediately under a Leitz fluorescence microscope with a 490 nm excitation filter and a 520 nm arrest filter. When cells were examined by CLSM, a 560 nm excitation filter and a 580 nm cut-off filter were used.

Virus infections

Vesicular stomatitis virus (VSV) (Indiana serotype) was propagated in BHK-21 cells according to Matlin et al. (1983). Adsorption of viruses was for 1 hour in DME-Hepes-BSA followed by a 2.5 hours growth period in DME-Hepes-FCS. For the exocytosis studies, Baf was added after the 1 hour adsorption period.

Electron microscopy

The bone slices were decalcified in 5% EDTA in 0.1 M phosphate buffer, pH 7.2. Cells were then postfixed in 1% OsO₄ in 0.1 M cacodylate buffer, pH 7.2, for 1 hour, dehydrated in acetone, and embedded in Epon LX 112. Ultrathin sections were cut with an LKB 2086 ultramicrotome and examined in a Philips 410 LS transmission electron microscope.

RESULTS

The RB of a resorbing osteoclast is enriched with both rab7 and lgp110

To identify the RB by immunofluorescence microscopy we performed double-stainings with an antibody to vitronectin receptor (αβ3). This protein has earlier been shown to cover both the basal plasma membrane and the RB of a resorbing osteoclast (Lakkakorpi et al., 1991, 1993). The lack of a recognizable resorption lacuna underneath and the lack of αβ3-positive RB were used as criteria of osteoclast inactivity. The lysosomal glycoprotein lgp110 was localized to membranes...
structures and distributed over the cytoplasm of inactive osteoclasts (Fig. 1a). In resorbing osteoclasts only a small fraction of lgp110 was detected in the perinuclear region and the majority of the protein was distributed over the RB (Fig. 1b-e), Rat osteoclasts, but not the non-osteoclastic mononuclear cells, were devoid of another lysosomal glycoprotein, lgp120 (Fig. 1f,g).

The two markers, lgp110 and lgp120, do not exclusively distinguish between lysosomes and late endosomes, since these proteins are also present on the membranes of late endocytic vacuoles (Geuze et al., 1988). Therefore, we studied the distribution of rab7, a small GTP-binding protein, which in earlier studies has been localized to CI-MPR-containing late endosomes but is not associated with lysosomal membranes in fibroblasts (Chavrier et al., 1990). In inactive osteoclasts the bulk of rab7 had a perinuclear distribution typical of late endosomes (Fig. 2a). In contrast, in resorbing osteoclasts the bulk of rab7 was associated with the RB and only a fraction of the protein had a perinuclear localization (Fig. 2b-f). Localization of rab7 to the RB indicates that this membrane has some endosomal characteristics.

Both lumenal and membrane-bound endocytic tracers are delivered to the RB

It could be expected that the RB receives material from cytoplasmic endosomes. Therefore, we next studied the pathways that various endocytic tracers use in osteoclasts. First, osteoclasts were incubated with HRP as a non-specific lumenal tracer and the distribution was studied by electron microscopy after the DAB reaction. Continuous incubation with HRP (or the other tracers used, see below) up to 3 hours did not affect the resorption activity, e.g. the maintenance of the sealing zone and the RB, of osteoclasts. After 1 hour incubation the HRP endocytosed by resorbing osteoclasts was localized to numerous, mostly perinuclear vesicles and vacuoles of various sizes (Fig. 3a). Typically these elements accumulated next to the RB (Fig. 3a,b,d). Generally, the DAB reaction product was also detected in the RB, showing that considerable amounts of HRP had accumulated in the extracellular lacuna (Fig. 3b-d). Occasionally, HRP-positive membranous structures were seen associated with the RB (Fig. 3d). These presumably represent HRP-containing elements that had just fused with the membrane.

We next studied the distribution of endocytosed human Fe-Tf, a marker of the early endosomal membrane recycling pathway, in rat osteoclasts. Osteoclasts were incubated with Fe-Tf, whereafter the tracer was immunolocalized. Generally, osteoclasts cultured on bone slices endocytosed much more Fe-Tf than mononuclear non-osteoclastic cells (or osteoclasts cultured on glass coverslips). When incubated with Fe-Tf for 60 minutes, the tracer was endocytosed into vesicular structures and also showed distinct, perinuclear staining (Fig. 4a,b). When endocytosis was allowed to proceed for 30 minutes, a significant fraction of Fe-Tf also reached the RB, co-localizing with vitronectin receptor (Fig. 4b, arrow, e,f; see also Fig. 6a). Co-localization of Fe-Tf with TfR was seen both in the
cytoplasmic vesicles and on the RB (not shown). The uptake of FITC-labeled Fe-Tf was inhibited when an excess of unlabeled Fe-Tf was present (not shown), also demonstrating that the endocytosis was a specific, receptor-mediated process. As a fraction of endocytosed Fe-Tf has been reported to reach late endosomes but not lysosomes (Stoorvogel et al., 1991), these results further indicate that the RB has some endosomal characteristics. Furthermore, the efficient delivery of membrane-bound Fe-Tf to the RB suggests that a significant membrane flow from the basal plasma membrane to the RB occurs via this pathway.

Recent studies with enveloped viruses have indicated that the basal plasma membrane of resorbing osteoclasts is not compositionally homogeneous but is divided into two membrane domains receiving either basolaterally or apically targeted viral glycoproteins, respectively (Salo et al., 1996). As TfRs are targeted basolaterally in epithelial cells (Fuller and Simons, 1986), we investigated the distribution of Fe-Tf/TfR complexes at the basal plasma membrane of osteoclasts. Osteoclasts were incubated with Fe-Tf for 60 minutes and the tracer was then immunolocalized in fixed, non-permeabilized cells. FITC-phalloidin was used to stain F-actin to identify osteoclasts maintaining tight contact, i.e. a sealing zone, with the bone surface (see Fig. 5). Clearly, Fe-Tf was not evenly distributed at the basal plasma membrane of osteoclasts maintaining bone attachment. There was, however, distinct variation in the distribution pattern of Fe-Tf between osteoclasts, which we presume correlates with different stages of the resorption cycle. Some osteoclasts had an even distribution of Fe-Tf at the peripheral domain of the plasma membrane, whereas the central region of the membrane was totally devoid of staining (Fig. 4g,j). Although these osteoclasts maintained bone-attachment they were not all necessarily associated with a lacuna underneath. Basolaterally targeted VSV G protein shows a similar polarized distribution at the plasma membrane of virus-infected, resorbing osteoclasts (Salo et al., 1996). However, in most cells the central region of the basal plasma membrane did not lack staining completely, but contained brightly stained patches, often distributed in a ring-like pattern (Fig. 4h,k). Finally, in some osteoclasts Fe-Tf displayed a patch-like pattern at the basal membrane without strict polarization to either peripheral or central domains (Fig. 4i,l). The latter two types of osteoclasts had a lacuna underneath. Inactive osteoclasts lacking a sealing zone had an even distribution of Fe-Tf at their plasma membrane (not shown).

Low concentrations of Baf rapidly inactivate osteoclasts without affecting the major pool of intracellular proton ATPases

We have earlier shown that Baf, a specific inhibitor of vacuolar-type proton ATPases, totally inhibits osteoclast resorption activity in vitro at concentrations as low as 3 nM (Sundquist et al., 1990). This effective concentration is remarkably low, since ≥30 nM is required to block proton ATPases in intact fibroblasts (Palokangas et al., 1994). One possibility is that Baf at low concentrations inhibits the maintenance of low pH in the resorption lacuna by

Fig. 2. The bulk of rab7 is distributed to the RB in active osteoclasts. Rab7 was immunolocalized using affinity-purified polyclonal antibodies and the distribution was studied by CLSM (0.25 μm-thick optical sections). In inactive osteoclasts rab7 has a vesicular, mostly perinuclear distribution (a). In resorbing osteoclasts lateral sections from the upper part of the cytoplasm (b) and from the level of nuclei (c) show perinuclear distribution of rab7, whereas the bulk of rab7 is localized to the RB (d) co-localizing with vitronectin receptor (e). (f) Corresponding lacuna.
specifically blocking proton ATPases on the RB (Baron et al., 1985; Blair et al., 1989; Väänänen et al., 1990). Alternatively, Baf may also affect osteoclast activity by blocking endocytic (Clague et al., 1994; Reaves and Banting, 1994; Chapman and Munro, 1994) and/or biosynthetic (Oda et al., 1991; Yilla et al., 1993; Palokangas et al., 1994) membrane traffic to the RB.

We adopted a protocol where rat osteoclasts, pre-cultured on bone slices for 48 hours, were treated with Baf for short time periods. The treatment of osteoclasts for 5 hours with 3 nM Baf did not have a significant effect on the maintenance of bone-attachment, i.e. a sealing zone, identified by the polarized, ring-like appearance of F-actin (Fig. 5c,e). However,

**Fig. 3.** Endocytosed HRP is delivered to the lacuna in resorbing osteoclasts. Osteoclasts were incubated with HRP (10 mg/ml) for 60 minutes at 37°C. Some vesicles containing DAB reaction product are distributed to the peripheral cytoplasm of resorbing osteoclasts (a), whereas the majority have perinuclear localization typically close to the RB (rb) (a,b). DAB reaction product is also found in the RB (b, short arrows; c) and in the resorption lacuna, where it covers the surface of bone matrix (bm) (b,c,d). Occasionally DAB reaction product can be found in the lumen lined by the RB representing HRP delivered to the lacuna (d, arrow). sz, sealing zone. Bars, 1 μm (a,b), 0.5 μm (c,d).
in the experiment shown in Fig. 5, only 11±5% of the osteoclasts, maintaining the sealing zone in the presence of 3 nM Baf (Fig. 5e, 5h 3 nM), were associated with a lacuna. In contrast, 56±9% of the control cells (Fig. 5e, 5h control) showed association with an underlying lacuna. Treatment of cells for 5 hours with 10 nM Baf totally inactivated osteoclasts, whereas bone-attachment was still maintained (Fig. 5e, 5h 10 nM). EM studies confirmed the maintenance of bone-attachment in Baf-treated osteoclasts, whereas only remnants of the RB, with abnormal morphology, were occasionally found in
Endocytosis in osteoclasts

these cells (Fig. 6c). Fe-Tf, endocytosed for 60 minutes, was localized by the immunoperoxidase technique to the RB in control osteoclasts (Fig. 6a), whereas only cytoplasmic organelles were Fe-Tf-positive in Baf-treated cells (Fig. 6c,d). These results indicate that treatment with 3 nM Baf for 5 hours is sufficient to efficiently inactivate resorbing osteoclasts. They also show that Baf inactivates osteoclast resorption activity without primarily affecting their bone-attachment. Thus, the maintenance of bone-attachment as such does not necessarily correlate with osteoclast resorption activity.

We further studied the distribution of acidic organelles in osteoclasts using the weak base Acridine Orange. The cells were incubated for 15 minutes with Acridine Orange and examined using either conventional fluorescence microscopy or CLSM. The incubations and rapid examination of the cells did not affect bone attachment. The lacuna under a resorbing osteoclast, cultured in vitro, did not show the intense accumulation of Acridine Orange, that has been reported earlier (Baron et al., 1985) (Fig. 7a-c). There were relatively few acidic vesicles in the cytoplasm of lacuna-associated osteoclasts, whereas the non-resorbing osteoclasts contained numerous acidic vesicles (compare Figs 7a and d). When osteoclasts were treated for 2.5 hours with 3 nM Baf no dramatic neutralization occurred and, instead, the number of cytoplasmic acidic vesicles in the lacuna-associated osteoclasts was increased (Fig. 8a). The exposure of osteoclasts for 5 hours to 3, 10 or 30 nM Baf did not neutralize their cytoplasmic vesicles either, although the treated osteoclasts were no longer associated with lacunae (Fig. 8c-e). Instead, the inactivated osteoclasts were loaded with acidic vesicles, similar to those in inactive untreated osteoclasts (Figs 7d, 8c-e). 100 nM Baf totally blocked cytoplasmic proton ATPases in osteoclasts, whereas a somewhat lower (30 nM) concentration was enough to block intracellular proton ATPases in the mononuclear non-osteoclastic cells (Fig. 8e,f). These results show that although low concentrations of Baf inhibit osteoclast resorption activity, they do not dramatically affect intracellular proton ATPases.

Low concentrations of Baf inhibit osteoclast resorption activity without affecting the biosynthetic pathway

We showed earlier that Baf treatment of fibroblasts inhibits the delivery of VSV G protein from the Golgi complex, presumably by neutralizing exocytic compartment(s) and inhibiting exocytic vesicle transport processes (Palokangas et al., 1994). As ongoing biosynthetic transport could be essential for biogenesis/maintenance of the RB (see Kornfeld and Mellman, 1989), it was of interest to study whether low concentrations of Baf affect exocytic transport in osteoclasts. Thus, osteoclasts were infected with VSV and, after the 1 hour adsorption period, the viruses were allowed to propagate for a further 2.5 hours in the presence of Baf at various concentrations. Finally, the cells were treated for 1 hour with cycloheximide in the presence of Baf to block further protein synthesis. In untreated
cells VSV G protein was delivered to the plasma membrane within 1 hour (Fig. 9a-c). The presence of 10 nM Baf did not affect the delivery of G protein from the Golgi complex to the plasma membrane, although osteoclasts were now inactivated and lost their lacuna association (Fig. 9d). Newly synthesized viral glycoproteins (VSV G protein, influenza hemagglutinin) or TfRs did not display polarized distribution on the plasma membrane of inactivated osteoclasts (not shown). However, 100 nM Baf, which totally blocked intracellular proton ATPases in osteoclasts (see Fig. 8f), also efficiently blocked

**Fig. 6.** Immunoperoxidase localization of endocytosed Fe-Tf in control and Baf-treated osteoclasts. Fe-Tf (0.1 mg/ml) was incubated with osteoclasts, either untreated or treated for 5 hours with 10 nM Baf, for 60 minutes at 37°C. Cells were fixed with McLean and Nakane’s periodate-lysine-paraformaldehyde fixative for 2.5 hours and Fe-Tf was localized by the immunoperoxidase technique (Brown and Farquhar, 1989) using polyclonal anti-transferrin primary antibodies and HRP-conjugated goat anti-rabbit secondary antibodies. In control osteoclasts Fe-Tf is localized to cytoplasmic vesicles, often seen as clathrin-coated vesicles next to the basal plasma membrane (a, small arrows; enlargement shown in b). Fe-Tf is also intensively distributed at the RB (rb) of resorbing osteoclasts (a, thick arrows). In Baf-treated osteoclasts sealing zones (sz) are still maintained, whereas the RB, if existing, is typically engulfed by osteoclasts and shows abnormal morphology (c, small arrows). Endocytosed Fe-Tf can be found in cytoplasmic endocytic vesicles, but not in the remnants of the RB (d, small arrows). Bars, 1 μm (a,c,d), 0.2 μm (b).
the exocytic transport of G protein (Fig. 9f). In mononuclear non-osteoclastic cells a somewhat lower (30 nM) concentration of Baf blocked exocytic transport (Fig. 9e), in accordance with the Acridine Orange data (Fig. 8e). In conclusion, low concentrations (≤10 nM) of Baf that inhibit osteoclast resorption activity did not affect biosynthetic transport in these cells.

**Early endosomes in osteoclasts are affected by low concentrations of Baf**

In fibroblastic cells the trans-Golgi network protein TGN38 can reach the plasma membrane, from which it is further retrieved to the TGN by endocytosis (Reaves et al., 1993; Bos et al., 1993). Neutralizing agents, such as chloroquine and bafilomycin, have been reported to block this recycling pathway and cause TGN38 to accumulate in endosomes (Chapman and Munro, 1994; Reaves and Banting, 1994). However, exposure of osteoclasts or bone-derived mononuclear non-osteoclastic cells for 2 hours to 100 nM Baf (or 100 μM chloroquine) did not change the localization of TGN38. In both drug-treated and control cells TGN38 was localized to the perinuclear area (see Fig. 10b,d,f,h), co-localizing with the Golgi-marker mannosidase II (not shown). Furthermore, in contrast to the results of Reaves et al. (1993) and Bos et al. (1993), we detected no endocytosis of antibodies directed against the luminal domain of TGN38. Therefore, either TGN38 does not reach the plasma membrane or only a minor pool of the protein cycles between the TGN and the plasma membrane in bone-derived cells.

A significant pool (~10%) of cellular CI-MPRs exists at the cell surface, from which they are continuously endocytosed and transported to the TGN (Braulke et al., 1987; Geuze et al., 1988; Klumperman et al., 1993). We verified the cycling of CI-MPRs from the osteoclast plasma membrane by following the endocytic uptake of CI-MPR-specific polyclonal antibodies from the culture medium (not shown). Subsequently, the cells were treated for 5 hours with various concentrations of Baf and the distribution of CI-MPRs and TGN38 was compared in osteoclasts and mononuclear non-osteoclastic cells. In untreated active osteoclasts, CI-MPRs had a perinuclear distribution identical to that of TGN38 (Fig. 10a,b). The RB in these cells was essentially devoid of CI-MPRs. In non-resorbing osteoclasts and mononuclear non-osteoclastic cells, CI-MPRs also had a perinuclear distribution, co-localizing with TGN38 (Fig. 10c,d). When osteoclasts were inactivated by treatment for 5 hours with 10 nM Baf, CI-MPRs partly maintained their perinuclear localization but, in addition, a pool of CI-MPRs were now found in peripheral, TGN38-negative organelles (Fig. 10e,f). These CI-MPR-containing peripheral organelles also contained endocytosed Fe-Tf, which indicates that they represent early endosomes (Fig. 10i,j). Treatment with 10 nM Baf also caused a similar change in the distribution of CI-MPRs in mononuclear non-osteoclastic cells (Fig. 10g,h). Higher concentrations (100 nM) of Baf, with shorter exposure times, gave identical results. Baf also caused a similar change in the distribution of CD-MPRs, which are highly expressed in osteoclasts (not shown).

In conclusion, endocytic processes in osteoclasts appear to be fairly sensitive to Baf. Blocking the endocytic pathway from the basal plasma membrane to the RB by Baf could affect the maintenance of the RB and thus explain the inhibition of osteoclast resorption activity at low concentrations of the drug.

**Fig. 7.** Distribution of organelles with low pH in osteoclasts. Cells were incubated with acridine orange (5 μg/ml) for 15 minutes at 37°C, washed, mounted in warm medium and investigated by either fluorescence microscopy with a 490 nm excitation filter and a 520 nm arrest filter (a,d) or by CLSM with a 560 nm excitation filter and 580 nm cut-off filter (0.25 μm-thick horizontal sections) (c,f). Relatively few acidic organelles appear in resorbing osteoclasts with an associated lacuna. Acidic organelles are distributed mainly in the perinuclear area, while no accumulation of acridine orange in the resorption lacuna was observed (a-c). Inactive osteoclasts are typically loaded with cytoplasmic acidic vesicles (d-f). Osteoclasts indicated by arrows. Bars, 10 μm.
DISCUSSION

The resorption lacuna formed by an activated osteoclast has been generally considered to be an equivalent of secondary lysosomes in other cells. Although this conclusion is relevant based on functional criteria, the composition of the RB facing the lacuna has remained poorly studied. The present results indicate that compositionally the resorption lacuna shares some characteristics with a late/mature endosome. Namely, both lgp110 and rab7, a small GTP-binding protein, were localized to the RB. Whereas lgp’s are present in both late endosomes and lysosomes (Geuze et al., 1988), rab7 has been shown to be associated exclusively with late endosomes of the degradative pathway in fibroblasts (Chavrier et al., 1990). Transport between early and late endosomes is specifically inhibited in cells expressing mutant rab7 (Feng et al., 1995), indicating that rab7 participates in vesicle fusion to late endosomes. CI-MPRs, another marker of late endosomes, have been localized in osteoclasts to vesicles in the vicinity of the RB (Baron et al., 1988). However, our studies show that the distributions of CI-MPRs and rab7 in resorbing osteoclasts are different. Rab7 is predominantly associated with the RB, whereas the bulk of CI-MPRs was localized to perinuclear structures, presumably the Golgi complex. In addition, Rabinowitz et al. (1992) reported that a phagocytic, tubulocisternal organelle in macrophages is rab7- and lgp-positive but contains CI-MPRs only at restricted domains of its membrane. The RB could thus represent a specialized membrane domain with late endosomal characteristics, where, according to the “vesicle shuttle model”, material from early endosomes would be transported in endosomal carrier vesicles along microtubules (Gruenberg and Howell, 1989; Gruenberg et al., 1989; Aniento et al., 1993). It is possible, however, that in resorbing osteoclasts rab7 participates in the terminal fusion of late/mature endosomes with each other and with the RB, a specialized “mixed” late endosomal/lysosomal membrane. This would also fit well with the “maturation model” supported by several investigators (Stoorvogel et al., 1991; Dunn and Maxfield, 1992; van Deurs et al., 1993).

The prominent association of rab7 with the RB in resorbing osteoclasts indicates that endocytic membrane transport is oriented towards this membrane. The fact that only a fraction of lgp110 was localized to cytoplasmic organelles further suggests that only a few cytoplasmic, terminal endocytic organelles exist in resorbing osteoclasts. Consistent with this, HRP, a lysosome-directed luminal endocytic tracer, was effi-
Endocytosis in osteoclasts

Endocytosed Fe-Tf/TfR complexes, which are normally retained in the early endosomal recycling pathway, also reached the RB. This adds further evidence to the special nature of the RB, as a fraction of Fe-Tf has been shown to appear in late endosomes, but not in lysosomes (Stoorvogel et al., 1991). Whether endocytosed HRP and Fe-Tf in resorbing osteoclasts are co-localized to cytoplasmic endosomes, whereas HRP is also delivered to cytoplasmic Fe-Tf-negative lysosomes in inactive osteoclasts, could be clarified by future double-endocytosis studies. If the proposed re-orientation of endocytic and biosynthetic pathways during osteoclast activation/inactivation indeed takes place, the accumulation of cytoplasmic, acidic organelles in either control or Baf-treated inactive osteoclasts could be due to the appearance of late endosomes/lysosomes with a more acidic luminal pH. However, at present we cannot exclude the possibility that many of the acidic organelles are not part of the endocytic pathway but are TGN-derived, proton ATPase-containing storage vesicles, which are ‘consumed’ during the resorption process. Related to this, Baron et al. (1990) showed re-orientation of the biosynthetic route towards cytoplasmic storage vacuoles in osteoclasts inactivated with calcitonin.

Newly synthesized VSV G protein was not delivered to the RB, indicating that this membrane is not the target for basolateral protein sorting. The finding that basolaterally targeted Fe-Tf/TfR complexes efficiently reached the RB, suggests that the route which Fe-Tf/TfR complexes follow bypasses the TGN where sorting occurs. It also suggests that the RB is equivalent to cytoplasmic late endosomes/lysosomes in other cells, rather than representing a specialized plasma membrane domain. However, cross-linking of VSV G protein at the basal plasma membrane by specific polyclonal antibodies resulted in its transport to the RB. This is consistent with the work of Gruenberg et al. (1989), showing that the delivery of G protein to terminal endocytic organelles can be triggered by antibody-mediated cross-linking of the protein at the plasma membrane.

The delivery of glycoproteins and proteolytic enzymes from the Golgi complex to the degradative pathway is essential for the biogenesis of lysosomes (Kornfeld and Mellman, 1989). Apart from the uptake of material for degradation, endocytosis as such may not be generally critical, as expression of mutant rab7 (blocking endosomal transport) has not been found to affect the maintenance of lysosomes (Feng et al., 1995). How the RB is formed and maintained throughout the resorption cycle has been unclear. However, the essential role of the biosynthetic pathway in RB biogenesis seems evident. Recent studies suggest that degraded bone matrix components are transcytosed from the RB.

Fig. 9. Low concentrations of Baf inhibit osteoclast resorption activity without affecting exocytic transport from the Golgi complex. Bone-derived cells were infected with VSV for 3.5 hours in control medium (a-c) or in the presence of 10 nM (d), 30 nM (e) or 100 nM (f) Baf. Cells were then treated for 1 hour with cycloheximide (50 μg/ml) in the presence of Baf before fixation and immunolocalization of VSV G protein. CLSM images (0.25 μm-thick sections) show that within 1 hour VSV G protein is transported to the plasma membrane in mononuclear non-osteoclastic cells (a) and in resorbing osteoclasts (b-c; lateral and horizontal sections). Exocytic transport in osteoclasts is also not affected by 10 nM Baf (d), whereas 30 nM (e) and 100 nM (f) Baf block the transport in mononuclear non-osteoclastic cells and in osteoclasts, respectively. n, nucleus.
to the basal plasma membrane for release (Salo et al., 1997; Nesbitt and Horton, 1997; Mostov and Werb, 1997). The removal of membrane from the highly convoluted RB due to transcytosis must be compensated. In fibroblasts endocytozed lipids are recycled efficiently via the early endosomal recycling pathway along with plasma membrane receptors (Dunn et al., 1989; Koval

---

**Fig. 10.** Baf affects endocytic processes in osteoclasts at low concentrations. Control and Baf-treated (5 hours, 10 nM) cells were double-immunostained for CI-MPR (a,c,e,g) and TGN38 (b,d,f,h) using specific polyclonal and monoclonal antibodies, respectively. CLSM images of 0.25 μm-thick sections are shown. CI-MPR and TGN38 co-localize in the perinuclear area in lacuna-associated osteoclasts (a,b) as well as in inactive osteoclasts (arrow) and in mononuclear non-osteoclastic cells (c,d). In the osteoclasts inactivated with 10 nM Baf, CI-MPRs are also localized in the peripheral organelles, which do not contain TGN38 (e,f). Similar redistribution of CI-MPRs in the presence of 10 nM Baf occurs in mononuclear non-osteoclastic cells (g,h). The peripheral CI-MPR-positive organelles (i, arrows) contain Fe-Tf endocytozed for 60 minutes into Baf-treated cells in the presence of the drug (j, arrows). This suggests that CI-MPRs retrieved from the plasma membrane are blocked by the drug in early endosomes. Bars, 10 μm.

---

**Fig. 11.** Schematic model showing the orientation of endocytosis during the osteoclast resorption cycle. (a) In inactive osteoclasts the endocytic pathway presumably consists of an array of cytoplasmic early endosomes (EE), late endosomes (LE) and lysosomes (LYS) characterized well in other cell types. (b) In resorbing osteoclasts endocytosis from the basal plasma membrane is targeted via early endosomes (black arrows) (or via early and late endosomes, open arrows) to the resorption lacuna, a late endosomal/lysosomal compartment. The biosynthetic pathway is also oriented towards the RB in resorbing osteoclasts. The proposed target site of Baf is indicated. SZ, sealing zone.
and Pagano, 1989; Mayor et al., 1993). Thus far, our attempts to follow the fate of endocytosed basal membrane in resorbing osteoclasts using labeled lipid probes have been unsuccessful. Although not quantitative, the immunofluorescence and immunoelectronmicroscopic studies show that a significant pool of endocytosed Fe-Tf/TfR complexes can reach the RB. This indicates that significant recycling of membrane from the basal surface to the RB occurs via endocytosis, thus balancing the transthyretic membrane traffic. Efficient endocytosis of Fe-Tf by osteoclasts may also be physiologically important, since Fe\(^{2+}\), released at the acidic pH of endosomes/resorption lacunae, could be essential for the enzymatic/free radical-mediated degradative processes of bone resorption.

Our studies show that in osteoclasts a pool of CI-MPRs reaches the plasma membrane and is further endocytosed. This is consistent with the results of studies showing that MPR pools in the Golgi complex, plasma membrane and late endosomes are at equilibrium (von Figura et al., 1984; Pfeffer, 1987) and that ~10% of the receptors are present at the plasma membrane in various cells (Braulke et al., 1987; Geuze et al., 1988; Klumperman et al., 1993). Plasma membrane CI-MPRs may function as receptors for lysosomal proteases and insulin-like growth factor II (Morgan et al., 1987; Kiess et al., 1988). In the case of a resorbing osteoclast this would mean economical uptake and reuse of secreted proteases. Consistently with this, the extracellular protease content increases when osteoclast bone resorption activity is increased (Vaes, 1988). If the endocytic membrane traffic is oriented towards the RB, endocytosed proteases can be delivered directly to the lacuna along this pathway and not via the TGN.

In resorbing osteoclasts newly synthesized VSV G protein and influenza virus hemagglutinin are transported to segregated peripheral and central domains of the basal plasma membrane, respectively (Salo et al., 1996). Here we show that Fe-Tf/TfR complexes, which are basolaterally targeted in epithelial cells (Fuller and Simons, 1986), also display a polarized distribution at the basal plasma membrane of resorbing osteoclasts. However, whereas ~70% of the lacuna-associated osteoclasts retained VSV G protein exclusively in the peripheral domain (Salo et al., 1996), the polarity of Fe-Tf/TfR complexes was not that strict. We propose that in resorbing osteoclasts endocytosed TRs transported to the RB can further follow the transthyretic pathway to the central ‘functional secretory domain’ in resorbing osteoclasts. Thus, osteoclasts in which Fe-Tf/TfRs are strictly polarized to the peripheral domain, presumably represent polarized cells without prominent transcytosis, i.e. they are in the very early or late phase of the resorption cycle.

The vacuolar proton ATPase inhibitor Baf provides an experimental tool to efficiently inactivate resorbing osteoclasts. Investigation of Baf effects was further motivated by the exceptional sensitivity of osteoclast bone resorption to this drug (Sundquist et al., 1990) and the recently suggested pharmacological differences between vacuolar-type proton ATPases in these cells (Chatterjee et al., 1992). We observed that transport from peripheral endosomes was dependent on an active endosomal proton ATPase both in osteoclasts and in non-osteoclastic mononuclear cells. This suggests that transport from early endosomes to late endosomes/TGN could be specifically affected, in accordance with previous reports (Clague et al., 1994; Chapman and Munro, 1994; Reaves and Banting, 1994). Our data do not exclude the possibility that the block occurs in peripheral late endosomes rather than in early endosomes, however (see van Weert et al., 1995; van Deurs et al., 1996). Although CI-MPRs retrieved from the plasma membrane were blocked in peripheral endosomes, no change in the distribution of TGN38 occurred. In parallel, we did not detect the appearance of TGN38 at the plasma membrane in osteoclasts, which is consistent with the results of Lippincott-Schwartz et al. (1991) with NRK cells, but contradicts the reports of others (Reaves et al., 1993; Bos et al., 1993). Although the maintenance of luminal acidic pH has been shown to be crucial for exocytic transport (Klioryski and Emr, 1989; Yilla et al., 1993; Palokangas et al., 1994), the endocytic pathway appeared to be more sensitive to Baf. Blockage of the endocytic traffic to the RB could fully explain the inhibition of osteoclast resorption activity by Baf.

In the present study we have used a number of approaches to characterize the composition and biogenesis of the RB membrane in resorbing osteoclasts. The strict pH- and temperature-sensitivities of osteoclast resorption activity have made many experimental approaches in the study of endocytosis unattainable. Interestingly, our results demonstrate that the RB shares some characteristics with late endosomal membranes, although compositional differences (such as the lack of lgp120) also exist. Although the life cycle of cytoplasmic late endosomes/lysosomes during the osteoclast resorption cycle still has to be characterized in detail, the present results suggest that both the endocytic and biosynthetic pathways play key roles in the dynamics and maintenance of the RB in resorbing osteoclasts (Fig. 11).

The authors thank Ms Sari Seinijoki, Mrs Minna Orreveläinen and Mr Eero Oja for technical assistance. Drs Karervo Metsikkö, Jari Salo, Päivi Lakkakorpi, David Keeling and Jean Gruenberg are acknowledged for discussions throughout the study, Dr Sinikka Eskelinen for advice in the transferrin studies, and Drs Jaakko Saraste and Bo van Deurs for critical reading of the manuscript. The study was supported by the Ministry of Education, Finland, the Academy of Finland and Biocenter, Oulu.

REFERENCES


Box, K., Wright, C. and Stanley, K. K. (1993). TGN38 is maintained in the trans-Golgi network by a tyrosine-containing motif in the cytoplasmic domain. EMBO J. 12, 2219-2228.


