

Evidence for the Presence of a Proton Pump of the Vacuolar H⁺-ATPase Type in the Ruffled Borders of Osteoclasts

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Abstract. Microsomal membrane vesicles prepared either from chicken medullary bone or isolated osteoclasts were shown to have ATP-dependent H⁺-transport activity. This activity was *N*-ethylmaleimide-sensitive but resistant to oligomycin and orthovanadate, suggesting a vacuolar-type ATPase. Furthermore, immunological cross-reactivity of 60- and 70-kD osteoclast membrane antigens with *Neurospora crassa* vacuolar ATPase was observed when analyzed by immunoblotting. Same antibodies labeled only osteoclasts in chicken and rat bone in immunohistochemistry. Im-

munoelectronmicroscopy localized these antigens in apical membranes of rat osteoclasts and kidney intercalated cells of inner stripe of outer medulla. Pretreatment of animals with parathyroid hormone enhanced the immunoreaction in the apical membranes of osteoclasts. No immunoreaction was seen in osteoclasts when antibodies against gastric H⁺,K⁺-ATPase were used. These results suggest that osteoclast resorbs bone by secreting protons through vacuolar H⁺-ATPase.

OSTEOLASTS are multinucleated giant cells that are responsible for bone resorption. Like secreting epithelial cells, they are polarized when active in bone resorption, showing three distinct specialized cell membrane areas (for review see Vaes et al., 1988). In addition to the basolateral membrane, which is rich in Na⁺,K⁺-ATPase (Baron et al., 1986), resorbing osteoclast exhibits a clear zone that mediates the attachment of resorbing cells to the bone matrix. The cytoplasm in the vicinity of this clear zone contains specialized cytoskeletal structures (Holtrop et al., 1974; Lakkakorpi et al., 1989). The third specialized membrane area, the ruffled border, faces the actual bone resorption site on the bone surface. The resorption lacuna underneath the ruffled border membrane is acidic. This has been shown by acridine orange accumulation experiments (Anderson et al., 1986; Barón et al., 1985) and direct micro-puncture measurements (Fallon, 1984). The acidic pH favors dissolution of the bone mineral. In addition, proteinases active at acid pH and capable of collagen degradation are present in osteoclasts (Vaes et al., 1988; Blair et al., 1986). Enzyme histochemistry suggests the presence of ATPase activity in the plasma membrane of the osteoclast (Akisaka et al., 1986). Baron et al. (1985) found at the ruffled border of the osteoclast a 100-kD lysosomal membrane polypeptide that showed immunological similarity to gastric H⁺,K⁺-ATPase. Now we report that osteoclasts contain an ATP-dependent proton pump that is clearly different from the gastric proton pump and from the mitochondrial proton pump but shows considerable immunological similarities to the vacuolar type H⁺-ATPase of *Neurospora crassa*.

Materials and Methods

Preparation of Bone Microsomes

Bone microsomes were prepared from medullary bone of regularly laying hens. Medullary bone from the tibia and femur was dissected out and immediately homogenized in a medium containing 5 mM Tris pH 7.4, 250 mM sucrose, 1 mM K₂CO₃, 1 mM DTT, and 1 mM EGTA in a glass-Teflon homogenizer. The homogenate was centrifuged at 1,000 g for 10 min. The supernatant was then centrifuged at 10,000 g for 30 min and the resulting supernatant centrifuged again at 100,000 g for 60 min. The final pellet was suspended in the homogenization buffer. Microsomes from chicken kidney medulla were prepared as described earlier for bovine kidney (Gluck et al., 1984). Gastric vesicles from pig stomach were prepared using the method of Saccomani et al. (1977).

Isolation of Osteoclasts

Osteoclasts were isolated from the medullary bone of regularly laying hens using an earlier described method (Hentunen et al., 1990) with minor modifications. Briefly, the tibias and femurs from three hens were quickly dissected on ice. Medullary bone was rinsed with PBS to remove bone marrow cells. Small pieces of the medullary bone were then incubated for 60 min at 37°C with gentle rotation in DME containing 5% FCS and 1.5 mg/ml collagenase (type I; Sigma Chemical Co., St. Louis, MO). After this digestion the bone pieces with suspended cells were passed through glass wool followed by centrifugation 400 g for 10 min. The pellet was suspended into 8 ml of DME containing 5% FCS and layered on the top of stepwise Percoll gradient (10, 20, 30, and 50% Percoll in isotonic sucrose). After centrifugation at 400 g for 25 min a band of osteoclasts visible between the first and the second interface was collected. Percoll was washed out with PBS by centrifugation. Small samples from final fractions were used to prepare smears that were stained by toluidine blue to count the number of mononuclear and multinuclear cells. Osteoclastic microsomes from isolated cells were prepared by homogenization in a glass homogenizer followed by centrifugation as described above for medullary bone microsomes.

Proton Transport Measurement

Proton transport by isolated membrane vesicles was assayed in a dual-beam, dual-wavelength spectrophotometer (UV-3000; Shimadzu Corp., Tokyo, Japan) by measuring the uptake of acridine orange. The membrane vesicles (35 or 70 μg protein) were suspended in 2 mM Hepes buffer, pH 7.4, containing 10 μM acridine orange, 2 mM MgCl_2 , 1 μM valinomycin, and 175 mM KCl in a final volume of 1 ml. The reaction was initiated by adding 1 mM Na_2ATP or Na_2GTP (pH was adjusted to 7.5 with Tris base) and the reaction was reversed by 1 μg of nigericin. 10 μM *N*-ethylmaleimide (NEM), 10 μM oligomycin or 100 μM orthovanadate were added before ATP when used. Acridine orange fluorescence was measured with excitation and emission wavelengths of 492 and 547 nm, respectively.

Immunoblotting

Antisera against the 57- and 67-kD subunits of *Neurospora crassa* vacuolar ATPase were generous gifts from Dr. B. Bowman (Department of Biology, University of California, Santa Cruz) (Bowman et al., 1988a,b).

Samples of isolated bone microsomes (25 μg protein) were fractionated by SDS-PAGE and transferred onto nitrocellulose (Towbin, 1979). Nitrocellulose strips were incubated with diluted primary antiserum or preimmunoserum (1:600) for 2 h at 37°C followed by washing with PBS. After a subsequent 30-min incubation (37°C) with swine anti-rabbit immunoglobulins, the strips were washed again with PBS and treated with peroxidase-rabbit antiperoxidase complex (Dakopatts, Copenhagen, Denmark). The reaction product was visualized using diaminobenzidine as an electron donor and H_2O_2 as a substrate.

Protein and Lysosomal Enzyme Measurements

Protein was measured by the method of Lowry et al. (1951). Acid phosphatase activity was measured with *p*-nitrophenyl phosphate as substrate at pH 4.5 (Bessey et al., 1946). Aryl-sulfatase was assayed using 40 mmol 2-hydroxy-5-nitrophenyl-sulfate as substrate at pH 5.3 (Roy, 1953). β -Glucuronidase was assayed using *p*-nitrophenyl-D-glucuronide as substrate at pH 5.3 (Kato et al., 1960).

Immunohistochemistry

Small pieces of chicken medullary bone or bone from the proximal tibia of 2-wk-old rats were fixed in ice-cold Carnoy fluid for 4 h and embedded in paraffin at 52°C in vacuum. 5- μm sections were stained using peroxidase-antiperoxidase method as described earlier in detail (Väänänen and Parvinen, 1983).

Immunoelectronmicroscopy

Trabecular bone slices from distal femurs of 5-d-old rats were fixed for 3 h either with 2% fresh paraformaldehyde containing 0.2% glutaraldehyde or 1% acrolein with 0.2% glutaraldehyde or 2% glutaraldehyde in phosphate buffer out of which paraformaldehyde preserved immunoreaction best. 10 5-d-old rats were given 7 μg parathyroid hormone (PTH)/10 g body wt s.c. (Sigma Chemical Co.) 2 h before being killed. Slices of kidney from normal male adult rats and gastric mucosa from fasted ones, were also fixed as described.

Ultracytometry was performed basically according to Tokuyasu (1973) with minor modifications (Laurila et al., 1989). Protein A-gold technique was used to label antigens (Roth et al., 1978; Geuze et al., 1981). The fixed tissue sections on the grids were first incubated in fluid gelatin and then transferred to PBS containing 0.02 M glycine at 37°C. From now on all the incubations were done at room temperature and washed with PBS in between. The grids were treated with 1% NaBH_4 and then incubated in normal sheep serum for 30 min. Antisera against 57- or 67-kD subunits of *Neurospora crassa* vacuolar H^+ -ATPase, non-immunosera, and rabbit antiserum against carbonic anhydrase III (Väänänen et al., 1986), used as a control hyperimmunoserum, were all diluted 1:100 in PBS, containing 1% sheep serum. Incubation time with primary antibodies was 1 h. Rabbit anti-mouse IgG were used as a bridge antibody with the monoclonal anti- H^+K^+ -ATPase antibodies (a generous gift from Dr. G. Sachs, Cure Foundation, Los Angeles, CA). Then they were incubated with protein A-gold complexes (5 or 10 nm) for 1 h (Slot and Geuze, 1985). After the immuno-

1. **Abbreviations used in this paper:** NEM, *N*-ethylmaleimide; PTH, parathyroid hormone.

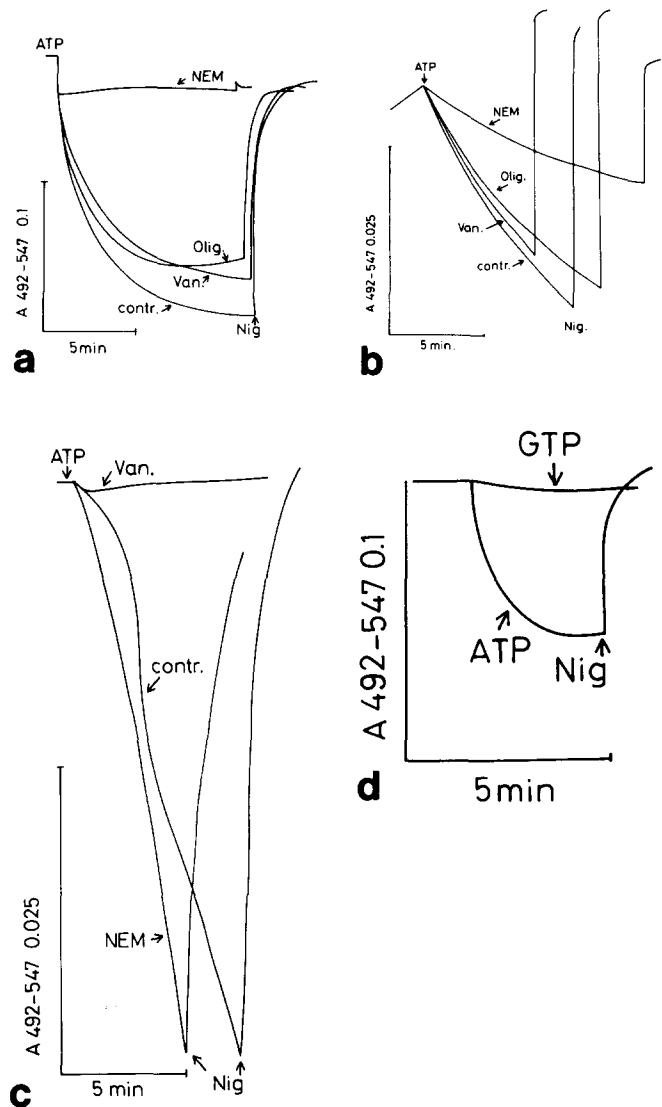


Figure 1. Proton transport in bone microsomes is ATP dependent (a and d) and inhibited by NEM, but not by orthovanadate or oligomycin. Kidney microsomes (b) showed similar inhibitor properties to bone microsomes, but gastric microsomes (c) were inhibited strongly by vanadate and unaffected by NEM.

staining the grids were counterstained with uranyl acetate and embedded in methyl cellulose. They were examined and photographed with Philips 410 LS transmission electron microscope. The effect of PTH treatment to the 60-kD subunit of vacuolar ATPase was further studied by quantitation of immunoreaction in ruffled border membranes of unstimulated and stimu-

Table I. Distribution of Lysosomal Enzymes in Different Centrifugation Fractions of Homogenized Medullary Bone

	Acid phosphatase		β -Glucuronidase		Arylsulfatase	
	SPA	%	SPA	%	SPA	%
1,000 g pellet	0.42	39	0.28	55	4.48	60
10,000 g pellet	3.50	23	1.12	14	20.55	19
100,000 g pellet	1.14	9	0.24	4	5.98	7
Final supernatant	0.78	29	0.37	27	2.70	14

Specific activities (SPA) and percentage of enzyme activity (%) in each fraction (nanomoles/minute per milligram protein).

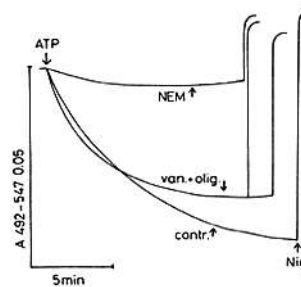
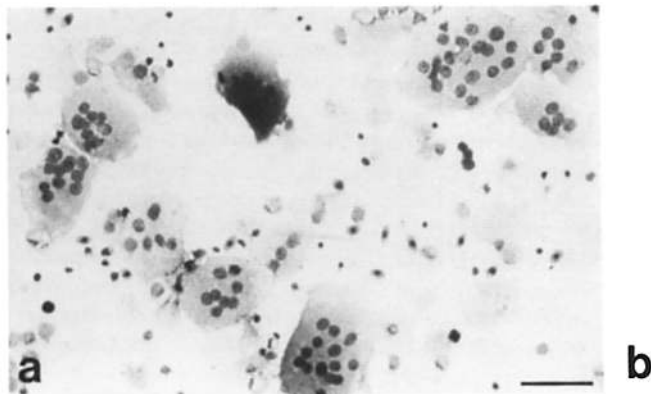


Figure 2. *a* shows fraction of isolated osteoclast from chicken medullary bone used to prepare microsomes for H^+ -transport measurements (*b*), which was sensitive to NEM and resistant to vanadate and oligomycin. Bar: (*a*) 30 μ m.

lated cells. The length of ruffled border membrane was measured from photographs and the number of gold particles was counted from corresponding areas.

Results

To study proton transport in osteoclast-derived membrane vesicles, a microsomal membrane fraction from chicken medullary bone was prepared. Fig. 1 *a* shows that acidification of the bone cell derived microsomes is ATP-driven and that the addition of the proton/potassium uncoupler nigericin (*Nig*) rapidly dissipates the pH gradient. Proton transport in bone microsomes is sensitive to NEM but insensitive to oligomycin and orthovanadate (Fig. 1 *a*). When acidification of microsomes derived from kidney was compared with that of the bone cell microsomes, the same pattern of inhibition effects with NEM and orthovanadate was observed (Fig. 1 *b*). In the contrary, proton transport in microsomes derived from the parietal cells of the gastric mucosa was abolished by orthovanadate but unaffected by NEM (Fig. 1 *c*).

We measured activities of some lysosomal enzymes in each fraction of differential centrifugation to evaluate possible lysosomal contamination in bone microsomes. The results are presented in Table I. Clear enrichment of lysosomal

enzymes is seen in the 10,000 *g* fraction that also revealed almost the same magnitude of proton transport activity with GTP as with ATP (ratio 1:1.8). Some activity of lysosomal enzymes was found in microsomal pellet (100,000 *g*) and when ATP was replaced by GTP, ITP or UTP, only minor proton transport activity was observed in this fraction (Fig. 1 *d*).

To test whether the microsomal fraction from medullary bone homogenate represented osteoclast-derived proton transport, we isolated osteoclasts from medullary bone (Fig. 2 *a*) and prepared microsomal fraction from this cell preparation. The percentage of nuclei in multinucleated cells varied between 70–85% from all counted nuclei in different experiments. These osteoclast-derived vesicles revealed the same characteristics of proton transport as did the vesicles from whole medullary bone microsomes (Fig. 2 *b*). We also prepared microsomal fraction from rat osteoblastic cell line UMR 106, using the same homogenization and centrifugation protocol as for bone and osteoclasts. No proton transport activity from these osteoblast-derived microsomes could be found (data not shown).

Western blotting of the bone cell microsome fraction was performed with antibodies against two subunits of *Neurospora crassa* vacuolar H^+ -ATPase and by antibodies against the gastric H^+ , K^+ -ATPase. Fig. 3 shows that the antiserum against the 57-kD subunit of *Neurospora crassa* H^+ -ATPase cross-reacted with a single polypeptide of 60 kD derived from the chicken bone cell microsomes. The antiserum against the 67-kD subunit of *Neurospora crassa* vacuolar H^+ -ATPase also recognized a polypeptide with a molecular mass of \sim 70 kD. Preimmunoserum gave negative staining reaction. mAbs raised against gastric H^+ , K^+ -ATPase did not show any labeling.

In sections of chicken medullary bone or rat trabecular bone the antiserum against the 67-kD subunit of *Neurospora crassa* vacuolar H^+ -ATPase selectively marked osteoclasts (Fig. 4). No clear evidence of staining was found in other bone cell types or in the bone marrow (Fig. 4, *a* and *b*). Control sections stained with preimmuneserum were negative (Fig. 4 *c*). The immunoperoxidase staining reaction in the chicken medullary bone osteoclasts was distributed diffusively throughout the cell. Thus no definite evidence for polarity of the immunostaining was obtained. Resorbing osteoclasts in the rat trabecular bone, however, showed clear

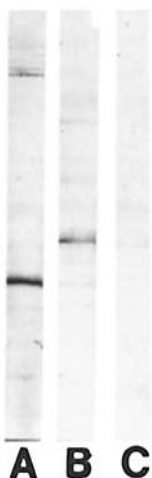


Figure 3. Immunoblots of bone microsomes stained with rabbit polyclonal antiserum raised against either the 57-kD (lane *A*) or the 67 kD (lane *B*) subunit of *Neurospora crassa* vacuolar H^+ -ATPase revealed protein bands of 60 and 70 kD, respectively. Lane *C* represents preimmunoserum to *B*.

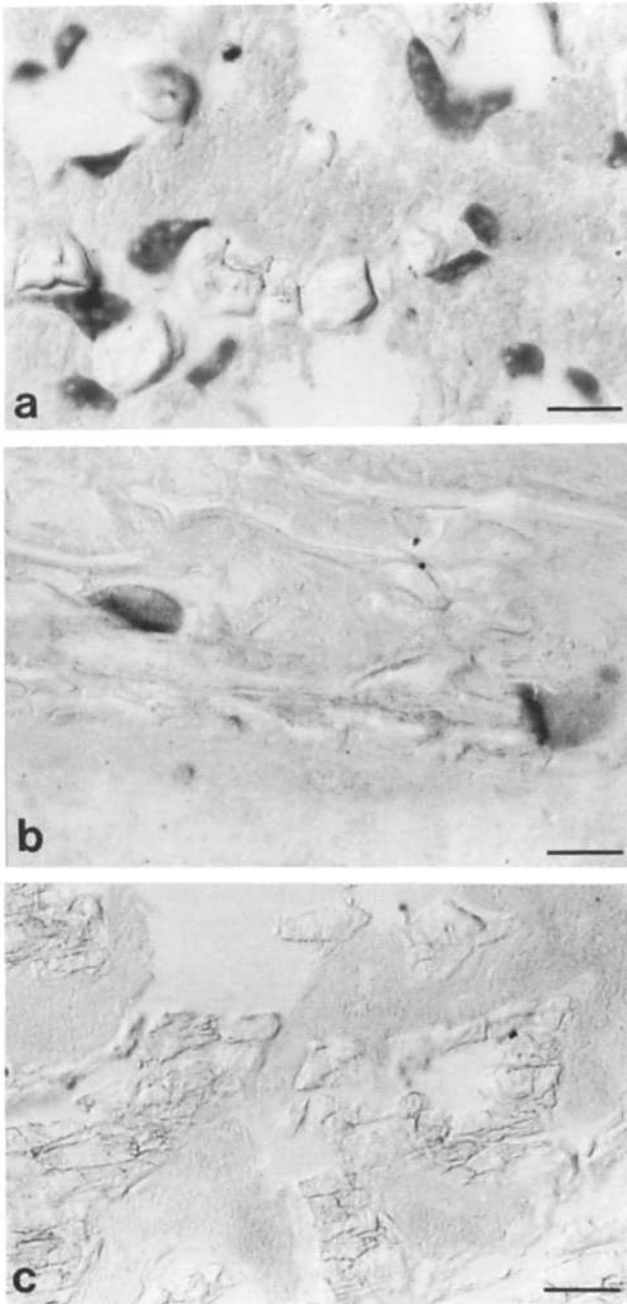


Figure 4. Immunohistochemical staining of chicken medullary (*a*) or rat trabecular bone (*b*) with antivacuolar H⁺-ATPase antisera showed specific labeling of osteoclasts with clear polarization of the reaction product to the ruffled border area, especially in the rat osteoclasts (*b*). Both sections (*a* and *b*) were stained with antiserum to the 70-kD subunit of *Neurospora crassa* vacuolar H⁺-ATPase. *c* represents preimmunoserum ($\times 320$). Bars, 30 μ m.

polarization of immunostaining to the area of the cell faced to the bone surface (Fig. 4 *b*). Immunostaining with antiserum against the 57-kD subunit of vacuolar H⁺-ATPase gave similar, but less intense, results to those stained with the antiserum against the 67-kD subunit.

In immunoelectron microscopy of osteoclasts, there was labeling at the ruffled border membrane when antiserum against either 57- or 67-kD polypeptide of *Neurospora crassa*

vacuolar ATPase was used (Fig. 5, *a* and *b*). Little labeling was found in the Golgi area or ER (Fig. 5 *g*). Secretory vesicles were labeled very faintly. The ampullary secretory vesicles showed a little stronger labeling than the small ones (Fig. 5, *e* and *f*) but still much less than the ruffled border membrane where most labeling was seen. Nuclei, lysosomes, or basal or healing zone plasma membranes or the extracellular matrix were totally devoid of immunoreaction (Fig. 5).

PTH treatment increased strongly both the 57- and 67-kD vacuolar H⁺-ATPase antibody reaction at the osteoclast ruffled border (Fig. 5, *c* and *d*). At the ruffled border area the increase of immunoreaction of 57-kD antigen compared with nontreated animals was from 0.84 ± 0.23 gold particles/ μ m ($n = 6$) to $1.49 \pm 0.58/\mu$ m ($n = 11$) ($P < 0.05$, *t* test). In other areas of osteoclasts, labeling remained similar to that in untreated osteoclasts.

When monoclonal antiserum against gastric H⁺K⁺-ATPase was used no labeling was seen in osteoclasts, not even at the ruffled border membrane (Fig. 6 *a*). Instead in rat parietal cells this antiserum gave intense labeling at the tubulovesicular and apical villus membranes (Fig. 6 *b*).

Kidney cells of the inner stripe of outer medulla, identified by ultrastructural criteria as intercalated cells, were densely labeled at the apical plasma membrane with antibodies against both 57- and 67-kD subunits of the vacuolar H⁺-ATPase of *Neurospora crassa* (Fig. 7 *a*). In this area of the kidney no labeling was seen at the basal membrane of the intercalated cells (Fig. 7 *b*). Neither was there any labeling seen in the principal cells.

Nonimmune serum and antiserum against carbonic anhydrase III used as controls did not give any labeling in osteoclasts, parietal cells or intercalated cells.

Discussion

Vacuolar ATPase represents a new class of proton pumps found in membranes of acidic compartments in many types of eukaryotic cells (Mellman et al., 1986). The similar type of proton pumping ATPase has been extensively characterized from the vacuolar membranes of *Neurospora crassa* (Bowman et al., 1988*a,b*). Two major polypeptides of molecular masses 70 and 60 kD are found in all vacuolar ATPases known so far and it has been previously shown that antibodies against these subunits show considerable cross-reactivity with comparable polypeptides of other vacuolar ATPases (Bowman et al., 1986; Mandala et al., 1986). Immunoblotting experiments from our isolated microsomal membrane fraction revealed also respective protein bands suggesting that immunologically related subunits are also found in chicken osteoclasts. This was further supported by immunohistochemistry, which revealed intense staining of chicken medullary bone osteoclasts. Immunohistochemistry also suggested that in bone, osteoclasts are the only cells with considerable amount of immunoreaction. Clearly polarized immunoreaction in rat osteoclasts and specific labeling at apical membrane, ruffled border, support the assumption that antibodies against 57- and 67-kD subunits of *Neurospora crassa* H⁺-ATPase also label H⁺-translocating ATPase in osteoclasts.

The significant increase of labeling in ruffled border membrane after PTH treatment is in further agreement that vacuolar type H⁺-ATPase plays an important role in bone

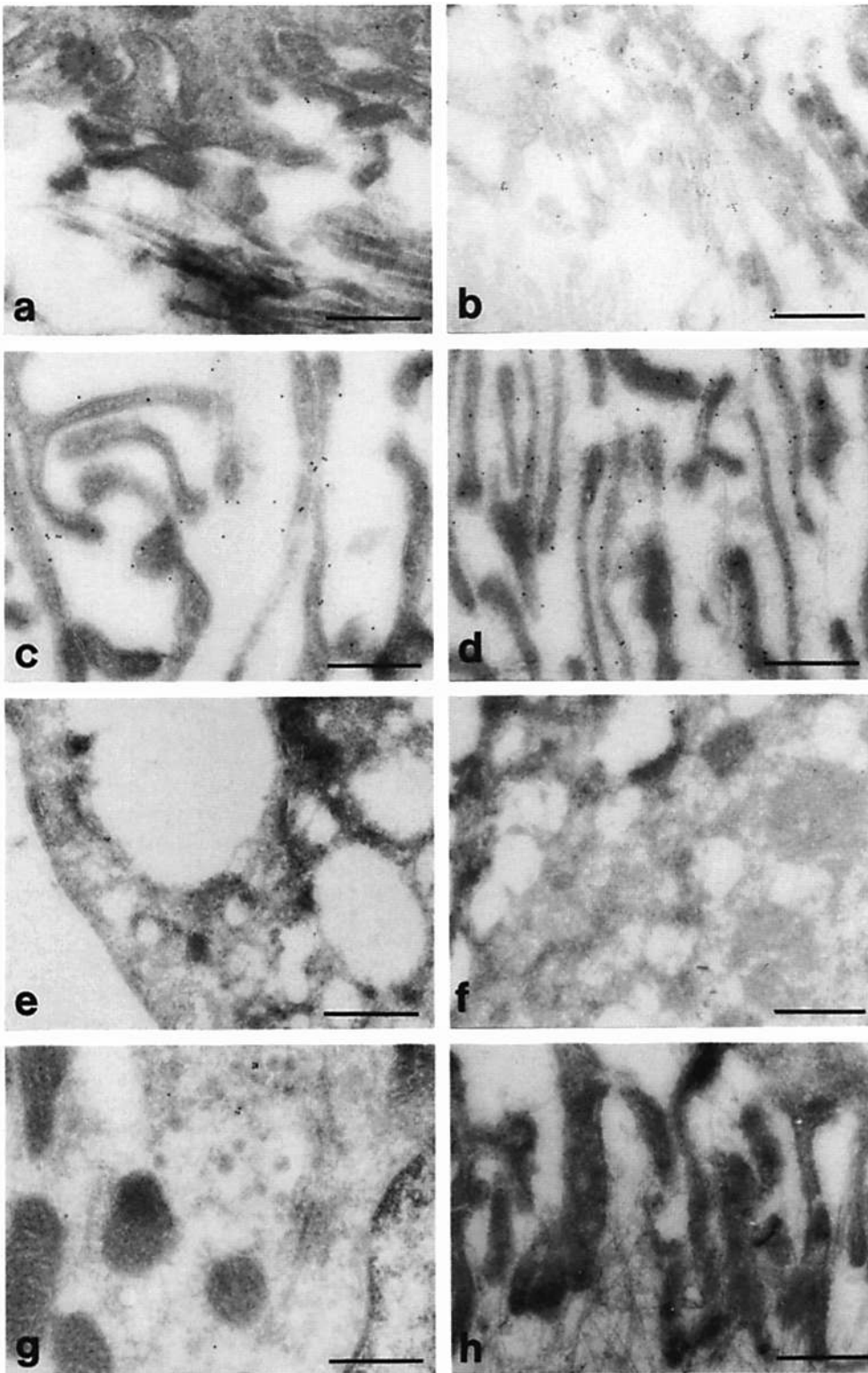


Figure 5. Immunoelectron microscopy of rat bone with vacuolar H^+ -ATPase antisera identified both the 67- (a) and the 57-kD (b) subunit at the ruffled border membrane area of the osteoclast. This staining was further enhanced by PTH treatment of the rats: immunolocalization of the 67- (c) and the 57-kD subunit (d). Weak staining reaction was observed in intracellular vacuoles and in Golgi vesicles but not in basolateral plasmamembrane: 57-kD antiserum (e-g). Hyperimmunoserum against carbonic anhydrase III used as an additional control showed no reaction (h). Bars, 1 μ m.

resorption. On the other hand, selective labeling of apical membrane of kidney intercalated cells of the inner stripe of outer medulla with *Neurospora crassa* vacuolar H^+ -ATPase antibodies supports the recent finding of Blair et al. (1989), that the proton pump in osteoclast is similar, if not identical, to the proton pump in kidney cells.

To characterize functional properties of osteoclastic pro-

ton pump, we isolated membrane vesicles from hen osteoclasts and studied their H^+ -transport activity. We used medullary bone of regularly egg-laying hens as a source of tissue because of its high number of osteoclasts. H^+ -transport properties of bone microsomal membrane fraction were compared with gastric microsomes and kidney microsomes that are known to contain H^+K^+ -ATPase and vacuolar AT-

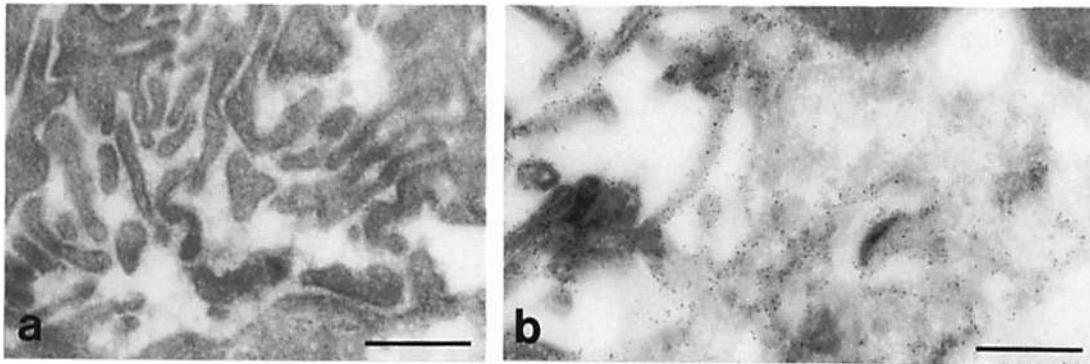


Figure 6. Antibodies against gastric H^+K^+ -ATPase did not show any labeling in the osteoclasts (a), but labeled intensively the apical membranes of rat gastric parietal cells (b). Bars, 1 μ m.

Pase, respectively (Saccomani et al., 1977; Gluck et al., 1984). Bone cell microsomes and kidney microsomes showed almost identical inhibition patterns with NEM and orthovanadate, whereas the microsomes derived from the parietal cells of the gastric mucosa showed profound differences. Also, in immunoelectron microscopy no reaction was seen at the apical membrane of the osteoclasts, whereas strong reaction was seen at the apical membrane of the parietal cells when monoclonal antibodies against gastric H^+,K^+ -ATPase were used. Thus, the possibility of an E_1E_2 type of proton pump being responsible for the acidification of the bone cell microsomes is ruled out.

A recent observation that ruffled border membrane of the osteoclasts and lysosomal membrane share the same 100-kD antigen, which also cross-reacts with H^+,K^+ -ATPase, suggested that extracellular lacuna can function as a large secondary lysosome and the proton pumps in lysosomes and at the ruffled border could be identical (Baron et al., 1985). As mentioned above our results with isolated bone microsomes and mAbs against H^+,K^+ -ATPase are against the presence of H^+,K^+ -ATPase in the ruffled border membrane. Lysosomal enzymes were clearly concentrated in the pre-microsomal pellet and vesicles from this pellet could use GTP almost as well as ATP as a substrate for H^+ -transport whereas microsomal pellet showed only traces of H^+ -transport with other substrates than ATP. Previous study (Harikumar and Reeves, 1983) has suggested that lysosomal proton pump can use GTP instead of ATP. However, the possibility that lysosomal fraction could convert GTP to ATP prevents us from

making firm conclusions about the substrate specificity of lysosomal H^+ -pump. Although our results clearly show that GTP could not replace ATP in the bone microsome preparation like in lysosome enriched pellet, we can't rule out the functional or structural identity of bone microsomal H^+ pump from the one present in lysosomes. So far, sequenced vacuolar ATPases from different sources have shown remarkable homology in their primary structures although some differences in subunit composition seem to occur (Nelson, 1989). Insensitivity of H^+ -transport in bone microsomes to oligomycin ruled out the possibility of marked mitochondrial contamination in our microsomal preparations.

The question how well our bone cell-derived microsomes represent osteoclasts needs some further comments. First, the fact that vacuolar ATPase antibodies detect only osteoclasts at tissue level and that both 60- and 70-kD antigens are enriched to our microsomal membrane fraction that shows also active proton transport of vacuolar type, support the conclusion that our H^+ -transporting vesicles are derived mostly from osteoclasts. Secondly, membrane vesicle fraction prepared from cultured osteoblast-like cells (UMR 106 cells) did not reveal any proton transport activity. Thirdly, our experiments with membrane vesicles derived from isolated, highly purified, osteoclasts revealed also NEM-sensitive and vanadate-resistant H^+ -transport, similar to that observed in medullary bone microsomes.

The selective labeling of the apical membranes of the osteoclast in trabecular bone and the above mentioned characteristics of proton transport in medullary bone cell-derived

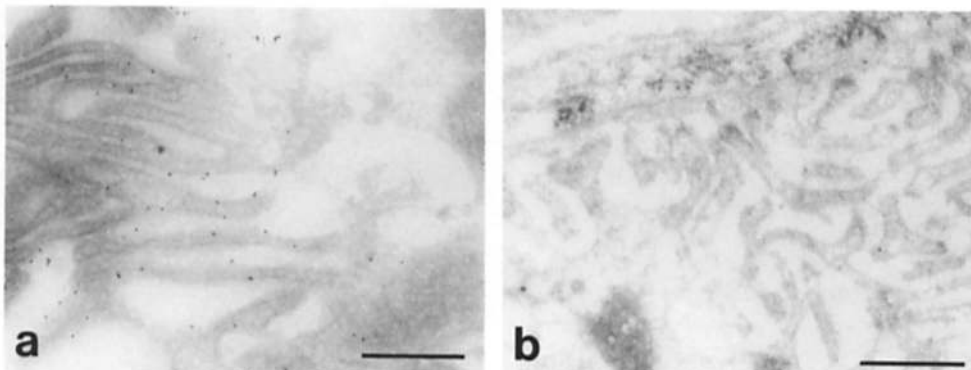


Figure 7. In rat kidney medulla antiserum against the 67-kD subunit of H^+ -ATPase labeled apical plasma membrane of intercalated cells (a), but not basolateral plasma membrane (b). Bars, 1 μ m.

microsomes strongly support the view that the observed active proton transport was associated with osteoclast derived membrane vesicles.

These data lead us to conclude that a vacuolar type H⁺-ATPase is located at the ruffled border membrane of the active osteoclast and is responsible for acidification of the extracellular resorption lacuna during bone resorption.

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