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THE OSTEOCLAST PROTON PUMP DIFFERS IN ITS PHARMACOLOGY AND CATALYTIC SUBUNITS FROM OTHER VACUOLAR H^+-ATPases

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Summary

Osteoclasts are multinucleated cells derived from the mononuclear phagocyte system in the hematopoietic bone marrow. Their function is to resorb bone during skeletal growth and remodeling. They perform this function by acidifying an enclosed extracellular space, the bone resorbing compartment. Analysis of proton transport by inside-out vesicles derived from highly purified chicken osteoclast membranes has revealed the presence of a novel type of multisubunit vacuolar-like H^+-ATPase. Unlike H^+-ATPases derived from any other cell type or organelle, proton transport and ATPase activity in osteoclast vesicles are sensitive to two classes of inhibitors, namely V-ATPase inhibitors [*N*-ethyl-maleimide (NEM) and bafilomycin A_1] and vanadate (IC_{50} 100 μmol l^{-1}), an inhibitor previously found to affect only P-ATPases. The osteoclast V-ATPase morphologically resembles vacuolar proton pumps and contains several vacuolar-like subunits (115×10^3, 39×10^3 and $16\times10^3 M_r$), demonstrated by Western blot analysis. Subunits *A* and *B* of the catalytic domain of the enzyme, however, differ from that of other V-ATPases. In osteoclasts, subunit *A* has an M_r of 63×10^3 instead of 67×10^3–70×10^3; in contrast, monocytes, macrophages and kidney microsomes, which contain a vanadate-insensitive H^+-ATPase, express the classical subunit *A* ($70\times10^3 M_r$). Moreover, two types of 57×10^3–$60\times10^3 M_r$ *B* subunits are also found: they are differentially recognized by antibodies and one is expressed predominantly in osteoclasts and the other in bone marrow cells and in kidney microsomes. Preliminary cloning data have indicated that the *B* subunit expressed in osteoclasts may be similar to the brain isoform. The osteoclast proton pump may, therefore, constitute a novel class of V-ATPase, with a unique pharmacology and specific isoforms of two subunits in the catalytic portion of the enzyme.

Introduction

Osteoclasts are multinucleated cells present in bone and responsible for bone resorption, a process necessary for bone growth and remodeling as well as bone repair. Several clinical entities involve an abnormal regulation of bone resorption which can be markedly increased, leading to local or systemic bone loss (bone tumors, osteoporosis, osteoarthritis), or which can be genetically deficient as in various types of osteopetrosis.

Key words: osteoclast, vanadate sensitivity, subunit *A* isoform, subunit *B* isoform.

The osteoclast is a highly motile cell which attaches to, and migrates along, the bone surfaces, mostly at the interface between bone and bone marrow (endosteum). It is usually a multinucleated cell formed by the asynchronous fusion of mononuclear precursors derived from the bone marrow and differentiating within the granulocyte–macrophage lineage. The osteoclast attaches to the mineralized bone matrix that will be resorbed by forming a tight ring-like zone of adhesion, the sealing zone. This attachment involves the specific interaction between adhesion molecules in the cell's membrane and some bone matrix proteins. The space contained inside this ring of attachment and between the osteoclast and the bone matrix constitutes the bone resorbing compartment. The osteoclast synthesizes several proteolytic enzymes which are then vectorially transported and secreted into this extracellular bone resorbing compartment. Simultaneously, the pH of this compartment is lowered by proton extrusion across its apical membrane (facing the bone matrix). The concerted action of the enzymes and the low pH in the bone resorbing compartment lead to the extracellular digestion of the mineral and organic phases of the bone matrix (Baron *et al.* 1985; Blair *et al.* 1989; Baron, 1989).

Based upon pharmacological and immunochemical data, it has been suggested that the H^+-ATPase present in osteoclast membranes is of the V-type, sensitive to NEM and bafilomycin A_1 but not to vanadate, and expressing the classical V_1 subunits *A* and *B* (Blair *et al.* 1989; Bekker and Gay, 1990; Vaananen *et al.* 1990).

The possibility that there are subtle differences in the properties and structure of mammalian V-ATPases has, however, been suggested (Nelson and Taiz, 1989; Forgac, 1989; Nelson, 1991; Wang and Gluck, 1990). These observations have led to the hypothesis that variations in isoforms of the multisubunit vacuolar proton pump(s) may constitute the basis for the differential targeting properties and for differential regulation of V-ATPases present in different organelles in the same cell, in different cells or in different organs (Nelson and Taiz, 1989; Forgac, 1989; Wang and Gluck, 1990).

Given the high degree of contamination of osteoclast preparations with vesicles derived from other cell types or from acidified intracellular organelles, we considered the possibility that indeed the pharmacology and structure of the ruffled-border osteoclast proton pump (OC V-ATPase) could differ from that of other proton pumps, but that these differences have remained undetected under the experimental conditions used in previous studies. Our results show that proton transport by osteoclast-derived vesicles has the unique property of being sensitive to inhibitors of two different classes of H^+-ATPases. Furthermore, two distinct isoforms of V-ATPase subunits *A* and *B* have been found in these preparations.

Materials and methods

Purification of osteoclast membranes

Osteoclasts were isolated from adult laying hens kept on a calcium-deficient diet for 14 days (Zambonin-Zallone *et al.* 1982), generating $8\times10^6\pm2\times10^6$ OCs per hen at a purity of 1:5±1.2 (osteoclast: contaminating cells). Owing to the measured difference in cell size, the purity of these preparations was calculated to be 90±4% osteoclast-derived

membranes. These preparations were resuspended in 5 vols of ice-cold HARM's buffer (10 mmol l^{-1} triethanolamine, 10 mmol l^{-1} acetic acid, 1 mmol l^{-1} EDTA, pH 7.4) containing 0.25 mol l^{-1} sucrose and protease inhibitors and passed 8–10 times through a 25 gauge needle. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant centrifuged at 10000 *g* for 10 min to pellet mitochondria. The 10000 *g* supernatant (4.5 ml per tube) was then layered over 5 ml cushions of 1 mol l^{-1} sucrose in HARM's buffer in 5 ml tubes and centrifuged at 100000 *g* for 30 min in a Beckman SW41 rotor. After centrifugation, 4.5 ml was aspirated, leaving the microsomal fraction in the 1 mol l^{-1} sucrose layer. This fraction was then diluted fourfould with HARM's buffer without sucrose and centrifuged at 100000 *g* for an additional 30 min. The resulting pellet was resuspended in HARM's buffer and washed by centrifugation at 100000 *g* for 30 min. This microsomal fraction (P1) was used in most experiments. In some experiments, the P1 fraction was further purified in 10 ml of 40% Percoll in a 15 ml tube and centrifuged at 28000 *g* for 15 min in a Sorvall SW34 rotor. A 3 ml fraction from the top of the gradient was removed, diluted five times with HARM's buffer (containing 0.25 mol l^{-1} sucrose, 1 μmol l^{-1} pepstatin and 2 μmol l^{-1} leupeptin) and centrifuged at 28000 *g* for 15 min to yield a purified membrane fraction (P2). These two membrane fractions were used for marker enzyme and proton transport assays, respectively. The different subcellular fractions were characterized by assaying marker enzymes. The results indicated a 9- to 11-fold enrichment in plasma membrane in the fractions with low levels of contamination by other organelles. The isolated osteoclast microsomal fraction (0.5–0.8 mg protein ml^{-1}) was resuspended in proton transport assay buffer, applied to Formvar-coated grids and negatively stained for 5–15 s with 1% phosphotungstate, pH 6.5, and examined using an electron microscope.

Proton transport assay

The proton transport assay was carried out essentially as described previously (Fuchs *et al.* 1989). Each assay was performed using 2 ml of membrane suspension (80 μg of protein in the case of P1 and 40 μg of protein in the case of P2 fractions) in the acidification buffer (150 mmol l^{-1} KCl, 5 mmol l^{-1} $MgSO_4$ and 20 mmol l^{-1} Hepes, pH 7.4 adjusted with tetramethylammonium hydroxide). To this membrane suspension, Acridine Orange (final concentration 1.5 μmol l^{-1}) and valinomycin (final concentration 0.5 μmol l^{-1}) were added and the samples were incubated at room temperature for 10 min in the presence or absence of various inhibitors. For the inhibition experiments, sodium orthovanadate was dissolved in distilled H_2O at a concentration of 0.5 mol l^{-1} and the pH adjusted to 7.4 with 0.5 mol l^{-1} HCl. The solution is then boiled for 2 min to destroy any polyoxyanions that are formed.

For the analysis of acidification by osteoclast microsomes at different substrate concentrations by the Eadie–Hofstee and the Hill methods, the assays were carried out as described above but with ATP concentrations ranging from 0.1 to 10 mmol l^{-1}.

Affinity purification of the OC V-ATPase

Purified IgG fraction from the anti $70\times10^3 M_r$ antibody of *Neurospora crassa* V-ATPase was coupled with CNBr-activated Affigel 10 (according to BioRad). The $C_{12}E_9$-

solubilized (1% w/v) microsomal fraction was passed through the column. The column was washed thoroughly with binding buffer (BioRad) and eluted with elution buffer (BioRad). The protein so obtained was neutralized with 1 mol l^{-1} Hepes, pH 9.0 buffer.

Assay of NEM-sensitive ATPase activity of the purified V-ATPase

Purified osteoclast V-ATPase (10 μl, 1 μg protein per assay) was incubated at room temperature for 10 min with 4 μl of phosphatidyl serine (0.5 μg ml^{-1} in 0.1% $C_{12}E_9$). Samples were diluted with distilled H_2O up to 50 μl in the presence or absence of 10 μmol l^{-1} NEM, vanadate or KNO_3 at different concentrations. The reaction was started with 50 μl of substrate solution (10 mmol l^{-1} $MgCl_2$, 80 mmol l^{-1} histidine, 1 mmol l^{-1} ouabain, 3 mmol l^{-1} azide, 5 mmol l^{-1} EGTA) and 10 mmol l^{-1} [^{32}P]ATP (0.5 mCi per 10 ml). After 30 min of incubation at 37°C, the reaction was stopped with 0.75 ml of perchloric acid, reacted with 0.25 ml of 5% ammonium molybdate solution and extracted with 2 ml of isobutanol + toluene (1:1) by vortexing for 15 s. The mixture was allowed to settled and the radioactivity in 100 μl of the organic phase was counted.

Immunoblot analysis

Microsomal lysates at three distinct stages of the osteoclast purification procedure were used to determine which subunits co-purified with the osteoclasts (osteoclast purity of 1:1000, 1:50 and 1:5). Polyclonal antibodies against the purified 115×10^3, 70×10^3, 60×10^3 and $39\times10^3 M_r$ subunits of chromaffin granule vacuolar proton pump (Wang *et al.* 1988), against the $16\times10^3 M_r$ subunit of plant vacuolar proton pump (Lai *et al.* 1988), against the 60×10^3 and $70\times10^3 M_r$ subunits of the *N. crassa* vacuolar proton pump (Bowman *et al.* 1988*b*) and against the *N. crassa* $70\times10^3 M_r$ subunit expressed as a fusion protein (W. Dschida and B. Bowman, unpublished results) as well as an antibody against a sequence-derived synthetic peptide from the $70\times10^3 M_r$ subunit of bovine coated-vesicle vacuolar pumps (Südhof *et al.* 1989) were used.

Peripheral blood monocytes (PBM) and bone marrow macrophages (BM) were prepared as described by Billecocq *et al.* (1990). Kidney microsomes were prepared according to Gluck and Caldwell (1987).

Results

Osteoclasts isolated from calcium-deficient laying hens (Zambonin-Zallone *et al.* 1982) were carefully processed to obtain highly pure preparations (>90% of the microsomal membranes were derived from osteoclasts) and the microsomal fraction was further purified, achieving a 10-fold enrichment in plasma membrane markers relative to the initial microsomal preparation. As illustrated in Fig. 1, most of the proton transport system(s) present in this microsomal fraction was derived from osteoclasts. ATP was found to be the only substrate for transport in the vesicles, confirming the low level of contamination with endocytic vesicles. High-magnification electron microscopy of negatively stained preparations showed high densities of ball-and-stalk structures, similar to those observed in kidney tubule apical membranes, which are enriched in V-ATPases (Brown *et al.* 1987), in 30–40% of the osteoclast microsome membranes. These

microsomal preparations were used to study the pharmacological properties of the proton transporter(s) and for immunochemical characterization of the enzyme subunits.

Pharmacologically (Fig. 2), inhibitors of V-ATPases inhibited 100% of the acidification by osteoclast membrane vesicles. The $K_{1/2}$ values for NEM, *N,N'*-dicyclohexylcarbodiimide (DCCD) and bafilomycin A_1 were 0.1 μmol l^{-1}, 35 μmol l^{-1} and 6 nmol l^{-1}, respectively. The mitochondrial V-ATPase inhibitors oligomycin, azide and fluoride had no effect (not shown). These data confirmed that, as reported by others (Blair *et al.* 1989; Bekker and Gay, 1990; Vaananen *et al.* 1990), the osteoclast proton pump exhibits properties of the vacuolar-type pumps, a finding in agreement with the high density of ball-and-stalk structures observed in electron micrographs of the osteoclast-derived microsomal vesicles used in this assay.

However, unlike any other V-ATPase, the OC V-ATPase could also be inhibited by vanadate, which blocked 100% of the acidification at a concentration of 1 mmol l^{-1} and with a $K_{1/2}$ of 100 μmol l^{-1} (Fig. 2). Most importantly, the sensitivity of proton transport to vanadate co-purified with the osteoclast membranes and, as expected for classical V-ATPases, was not detected in kidney microsomes derived from the same animals, eliminating the possibility of an artefact or of species specificity. To determine whether two H^+-ATPases were present in these osteoclast-derived microsomal preparations, analysis of H^+ transport as a function of ATP concentration by the methods of Hill and Eadie–Hofstee was performed. As shown in Fig. 3, the results demonstrated the presence of a single K_m for ATP (800 μmol l^{-1}) with a Hill coefficient of 0.9, suggesting that only one type of H^+-ATPase was present and consistent with the reported K_m for other H^+-ATPases.

Studies performed on the partially purified enzyme confirmed the sensitivity to vanadate and the presence of only one NEM-sensitive ATPase in these preparations. The specific activity of the purified enzyme was found to be 85 ± 5-fold higher than that of the

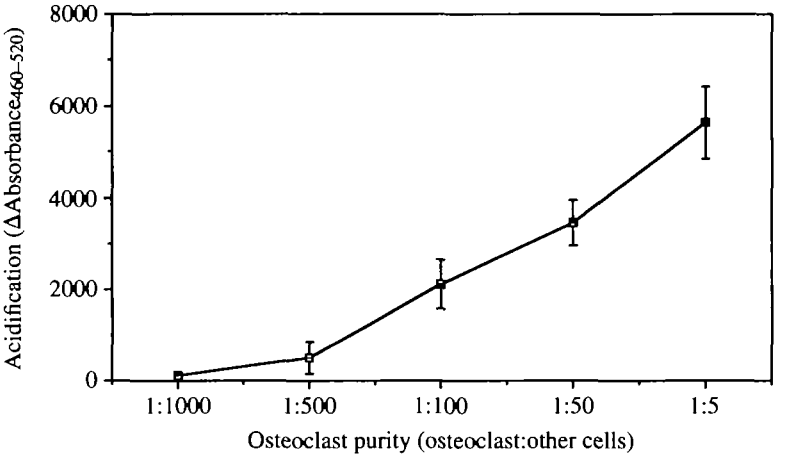

Fig. 1. Purified osteoclast membranes contain F_1F_o-like structures and a very efficient H^+-ATPase. Mean ± S.D. of 4–6 determinations of ΔpH per μg protein as a function of osteoclast purity. These results demonstrate that proton transport in these preparations is mostly associated with osteoclast membranes and not with contaminating cells.

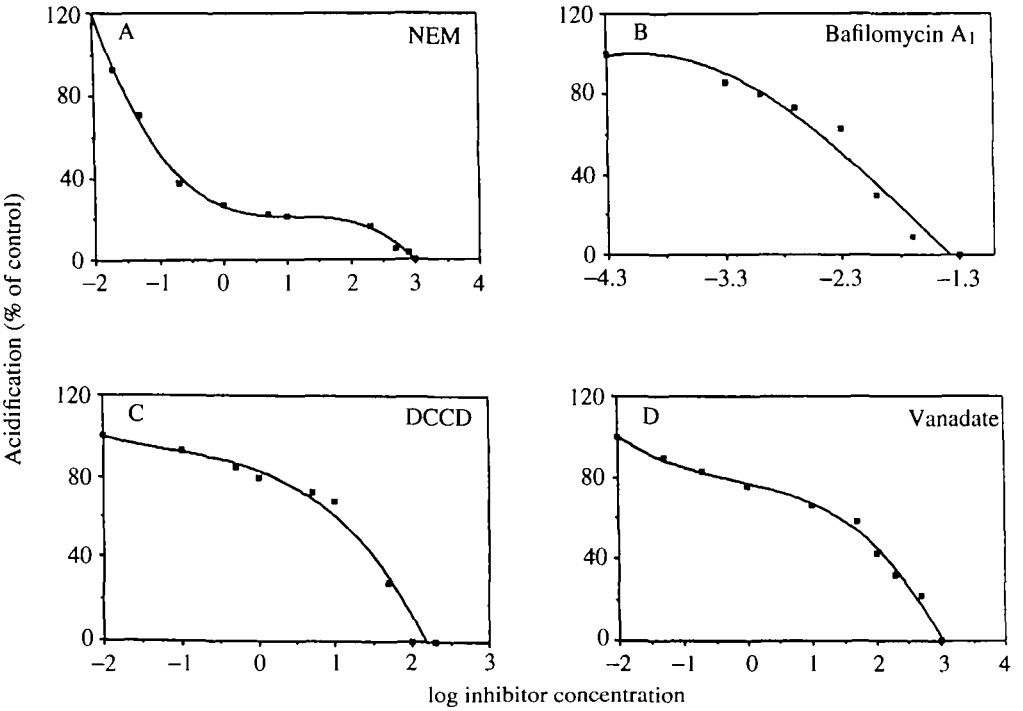

Fig. 2. The osteoclast proton pump is inhibited by the classical inhibitors of V-ATPases but also by vanadate, defining it as a new class of V-ATPase. Microsomal fractions from osteoclast (40 μg per 2 ml) and kidney (100 μg per 2 ml) were preincubated in the acidification buffer with the indicated doses (measured in μmol l^{-1}) of NEM (A), DCCD (B), bafilomycin A_1 (C) and sodium orthovanadate (D) for 10 min at room temperature and acidification was carried out as described in the text. All the inhibitors completely blocked the acidification with half-maximal concentrations ($K_{1/2}$) for DCCD, NEM, bafilomycin A_1 and vanadate of 35 μmol l^{-1}, 0.1 μmol l^{-1}, 6 nmol l^{-1} and 100 μmol l^{-1}, respectively.

crude homogenate. Assay of NEM- and vanadate-sensitive V-ATPase activity was carried out after affinity purification of the enzyme, using antibodies against the $70\times10^3 M_r$ subunit of the *N. crassa* vacuolar pump, and in the presence of 5 mmol l^{-1} NEM and 1 mmol l^{-1} vanadate. Only one K_m for ATP was found (0.9 mmol l^{-1}) and the V_{max} of the reaction was 7.6±0.3 μmol min^{-1} mg^{-1} protein. Interestingly, and further supporting the fact that the OC H^+-ATPase differs from other V-ATPases, the pH optimum of the enzyme was of 5.8, instead of 6.8–7.2 for other reported V-ATPases. As observed for proton transport, the ATPase activity could be inhibited by vanadate, and in the same range of concentrations (IC_{50}=100 μmol l^{-1}).

To analyse the immunological relationship of the OC V-ATPase to other V-ATPases, antibodies against several vacuolar proton pump subunits were used in immunoblots of osteoclast membrane proteins obtained at different stages of purification. Antibodies against the $115\times10^3 M_r$, 60×10^3 and $39\times10^3 M_r$ subunits of the chromaffin granule V-ATPase (Wang *et al.* 1988) and the $16\times10^3 M_r$ DCCD-binding proteolipid of a plant V-ATPase (Lai *et al.* 1988) detected proteins of the expected relative molecular masses,

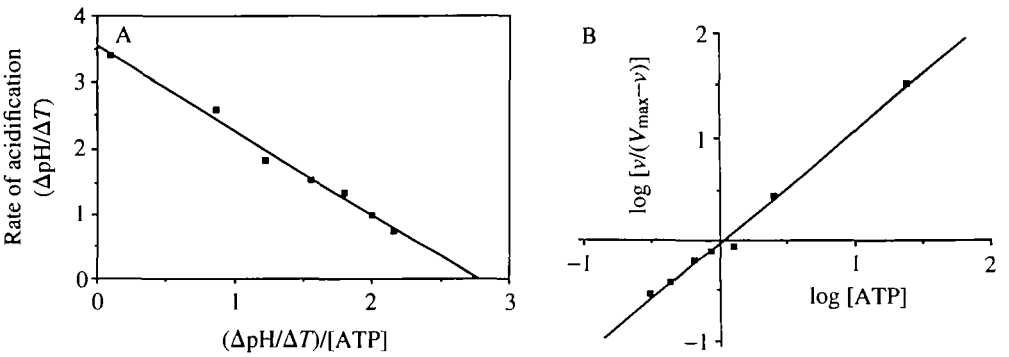

Fig. 3. Analysis of acidification by osteoclast microsomes at different substrate concentrations by the Eadie–Hofstee (A) and the Hill (B) methods. Only one K_m for ATP was obtained (800 μmol l^{-1}) and the Hill coefficient was 0.9, demonstrating the presence of a single ATP binding site for V-ATPases in these membranes.

which co-purified with the osteoclasts (Fig. 4), further confirming the vacuolar-like nature of the OC V-ATPase. In contrast to the 60×10^3M_r subunit mentioned above, another 60×10^3M_r subunit was recognized by antibodies against the *N. crassa* ATPase (Bowman *et al.* 1988*b*) in unpurified fractions and it decreased in amount with osteoclast purity. Bone marrow macrophage preparations were found to contain a mixture of both forms, but kidney microsome preparations expressed predominantly the isoform recognized by the antibodies to *N. crassa* and osteoclasts contained predominantly the isoform recognized by antibodies to chromaffin granule subunit *B*. These results demonstrate the existence of two distinct isoforms of subunit *B* in cells from the same species: a 60×10^3M_r subunit predominantly associated with the OC V-ATPase and another 60×10^3M_r subunit predominantly expressed in kidney microsomes and in other cells.

Similarly, the 70×10^3M_r protein detected by antibodies against the 70×10^3M_r catalytic subunits from chromaffin granules (Moriyama and Nelson, 1988), coated vesicles (Südhof *et al.* 1989) or *N. crassa* (Bowman *et al.* 1988*a*) was present in unpurified fractions and *decreased* with osteoclast purity (Fig. 4), suggesting that this isoform of subunit *A* was mostly present in contaminating cells. Most interestingly, the antibodies against the *N. crassa* V-ATPase 70×10^3M_r subunit also detected a second protein, with an M_r of approximately 63×10^3, which was undetectable in unpurified fractions, but appeared and progressively *increased* in amount during osteoclast purification (Fig. 4). The immunological similarity of this 63×10^3M_r polypeptide to the *N. crassa* 70×10^3M_r subunit was confirmed by immunoblotting with antibodies raised against the *N. crassa* protein expressed in *Escherichia coli* as a recombinant fusion protein (a gift from W. Dschida and B. Bowman). The 63×10^3M_r protein was also undetectable in microsomes from kidney, bone marrow macrophages or peripheral blood monocytes. The 70×10^3, 63×10^3 and 60×10^3M_r subunits could be distinguished by their electrophoretic mobility when blotting with the antibodies to the *N. crassa* 70×10^3M_r and chromaffin granule 57×10^3M_r subunits simultaneously. The possibility that this 63×10^3M_r band

resulted from proteolytic degradation in the osteoclast preparations was also ruled out by the observation that processing the unpurified fraction (1/1000) for up to 6 h under the conditions of osteoclast purification did not induce any decrease in the amount or M_r of the 70×10^3M_r subunit, nor did it induce the appearance of a 63×10^3M_r band. Hence, subunit *A* may also exist in two isoforms, a 63×10^3M_r predominantly expressed in osteoclasts and a ubiquitous 70×10^3M_r isoform.

In screening a chicken osteoclast cDNA library, we have recently isolated a clone encoding a *B* subunit isoform which had the same immunological properties as the *B* subunit expressed in osteoclasts. Analysis of the deduced amino acid sequence showed

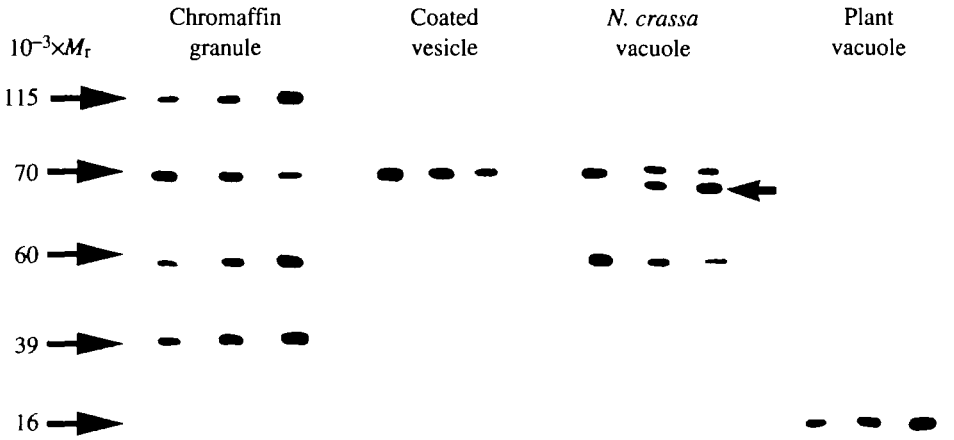

Fig. 4. Immunoblot analysis of the subunit composition of the osteoclast V-ATPase reveals a new and specific p63 isoform of the catalytic 70×10^3M_r subunit Immunoblot analysis of microsomal lysates at three distinct stages of the osteoclast purification procedure were performed to determine which subunits co-purified with the osteoclasts (osteoclast purity of the left, middle and right panels were 1:1000, 1:50 and 1:5, respectively). Because the purified osteoclasts have a volume approximately 50–100 times larger than that of the contaminating cells, the 400-fold purification achieved in the ratio of osteoclasts to other cells represents an approximately 30-fold purification in terms of total membrane, thereby explaining the relatively small increment in specific proton pump polypeptides during osteoclast enrichment. The amounts of the 115×10^3, 60×10^3 and 39×10^3M_r subunits of vacuolar proton pump (recognized by antibodies against the corresponding subunits of chromaffin granule vacuolar pump) as well as the 16×10^3M_r DCCD-binding subunit (recognized by antibody against the 16×10^3M_r subunit of the plant vacuolar proton pump) increased with osteoclast purity, demonstrating their presence in osteoclasts and the multisubunit nature of the OC V-ATPase. In contrast, the amounts of the 60×10^3M_r subunit (recognized by antibody against the 60×10^3M_r subunit of the *N. crassa* vacuolar proton pump) and the 70×10^3M_r subunit (recognized by antibody against the 70×10^3M_r subunits of chromaffin granules, *N. crassa* and coated vesicle vacuolar pumps) were found to decrease with osteoclast purity, suggesting that they were mostly present in contaminating cells. Interestingly, a 63×10^3M_r band (arrow) was recognized by antibodies to the 70×10^3M_r subunit of the *N. crassa* vacuolar pump and appeared progressively during osteoclast purification.

more than 90% identity with other *B* subunit sequences in the central region of the molecule but diverged at both the N- and C-terminal ends from the *N. crassa*, bovine or human kidney isoforms. Two papers, however, recently reported a second isoform of subunit *B* (Puopolo *et al.* 1992; Nelson *et al.* 1992), preferentially expressed in brain, which our sequence resembles more closely. We have also generated, using the polymerase chain reaction, a fragment of the chicken subunit *A* and isolated several clones from the osteoclast library which, at present, all encode *A*-like sequences. Whether one of these could be encoding a different isoform has not yet been established.

Discussion

The data presented here suggest the existence of two different isoforms of each of the two subunits forming the catalytic portion of these pumps: a $63\times10^3 M_r$ isoform (p63) of the $67\times10^3-70\times10^3 M_r$ subunit *A* and two isoforms of the $57\times10^3-60\times10^3 M_r$ subunit *B*, which are differentially recognized by antibodies to the *N. crassa* and bovine chromaffin granule subunits. Both the $63\times10^3 M_r$ subunit *A* and the subunit *B* specifically recognized by antibodies to the chromaffin granule 57×10^3–$60\times10^3 M_r$ subunit *B* are highly, and possibly specifically, expressed in osteoclast membranes.

In the same preparations as those used for analysis of the subunit composition of the V-ATPase, proton translocation showed a unique pharmacological profile. Proton transport in osteoclast-derived membranes was not only sensitive to NEM and bafilomycin A_1, as are classical vacuolar proton pumps, but also to vanadate, an inhibitor of P-ATPases. The concentration of vanadate required to inhibit the osteoclast proton pump was, however, much higher than that required to inhibit P-ATPases. The molecular basis for the vanadate sensitivity of this proton pump, possibly defining it as a new class of multisubunit V-ATPase, still remains to be determined. It is, however, tempting to speculate that the osteoclast H^+-ATPase has acquired its unique pharmacological profile through the expression of the specific isoform(s) of the proton pump subunits *A* and *B* mentioned above: together, these two subunits form the catalytic portion of the enzyme and this is the site at which vanadate would be expected to inhibit the ATPase if it were to act directly on the pump.

Although this putative new class of V-ATPase may be expressed in several cells and/or organelles, their level of expression does not yet allow their identification. This novel type of V-ATPase might, therefore, be expressed in a cell-specific manner, which would provide a potential way for cell-specific targeting of therapeutic interventions in diseases involving an increased resorption of bone or cartilage, such as osteoporosis and osteoarthritis. Current efforts at purifying this enzyme, developing specific antibodies and cloning its various subunits might provide tools to test these possibilities further.

Because of the novel and apparently unique pharmacology of this proton pump, several possibilities were considered. The possibility that these membranes could contain two types of proton pump, a V-ATPase conferring the NEM and bafilomycin sensitivities and a P-ATPase conferring the vanadate sensitivity, was ruled out on the basis of several experimental results. First, it is possible to inhibit 100% of proton transport with each inhibitor used separately (Fig. 2), which strongly argues against the presence of two types

of pumps or two types of acidifying vesicles in our microsomal preparations. Second, analysis of H^+ transport by inside-out vesicles in the presence of various concentrations of ATP demonstrated the presence of only one K_m for ATP in these preparations (Fig. 3A), a very unlikely result if indeed two H^+-ATPases were present in these membranes. Third, immunoblot analysis with antibodies raised against the two P-ATPases that are sensitive to vanadate, the *N. crassa* plasma membrane electrogenic pump (Aaronson *et al.* 1988; Mandala and Slayman, 1989) and the gastric H^+/K^+-ATPase (R. Baron, L. Neff and M. Caplan, unpublished results), did not recognize any protein in our microsomal preparations or *in situ*. Fourth, both the morphological and the pharmacological data (presented in Figs 1C and 2, respectively) demonstrate a high concentration of multisubunit, V-like proton pumps in these membranes. Hence, only one class of V-ATPase seems to be responsible for H^+ transport in these osteoclast-derived vesicles.

Differences in enzymatic properties and structure have previously been reported among various subclasses of V-ATPases (Forgac, 1989). Differences in K_m for ATP and in substrate specificity are found, for instance, between the kidney brush border and microsomal ATPases (Wang and Gluck, 1990) and between endosomes and lysosomes (Yamashiro *et al.* 1983; Ohkuma *et al.* 1982). Such differences have been attributed to variations in structure of the pumps and, in particular, to the existence of two isoforms of subunit *B* (Gluck and Caldwell, 1987; Wang and Gluck, 1990), given the fact that only one form of catalytic subunit *A* has been reported so far (Wang and Gluck, 1990).

Our data provide strong immunochemical evidence for the existence of two isoforms of both subunit *A* and subunit *B*. For subunit *B*, our data confirm the biochemical data of Wang and Gluck (1990), who reported the expression of two isoforms of subunit *B* present in different membrane fractions in the kidney. However, and in contrast with their finding, we found that each isoform of subunit *B* is preferentially associated with distinct cell populations: one is predominantly expressed in osteoclast membranes and the other in kidney membranes. Even more intriguing is our finding of two isoforms of subunit *A*, a $67\times10^3-70\times10^3 M_r$ 'classical' form expressed in all cells and a novel $63\times10^3 M_r$ isoform, which we found only in preparations containing high concentrations of osteoclast membranes. Most importantly, subunit *A* is the site of the catalytic ATP-binding site (Forgac, 1989) and, by analogy with the mitochondrial F_1F_o-ATPase, subunit *B* may participate in catalysis (Ysern *et al.* 1988; Futai *et al.* 1988). One could therefore speculate that the variations in isoforms making up the catalytic portion of the osteoclast V-ATPase, involving subunits *A*, *B* or both, may explain the unique sensitivity of this pump to NEM, bafilomycin A_1 and vanadate.

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CHAPTER 6. V-ATPases and ion channels of kidney vacuoles and plasma membranes