The a3 Isoform of the 100-kDa V-ATPase Subunit Is Highly but Differentially Expressed in Large (≥10 Nuclei) and Small (≤5 Nuclei) Osteoclasts*

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Osteoclasts dissolve bone through acidification of an extracellular compartment by means of a multimeric vacuolar type H+-ATPase (V-ATPase). In mammals, there are four isoforms of the 100-kDa V-ATPase “a” subunit. Mutations in the a3 isoform result in deficient bone resorption and osteopetrosis, suggesting that a3 has a unique function in osteoclasts. It is thus surprising that several studies show a basal level of a3 expression in most tissues. To address this issue, we have compared a3 expression in bone with expression in other tissues. RNA blots revealed that the a3 isoform was expressed highest in bone and confirmed its expression (in decreasing order) in liver, kidney, brain, lung, spleen, and muscle. In situ hybridization on bone tissue sections revealed that the a3 isoform was highly expressed in multinucleated osteoclasts but not in mononuclear stromal cells, whereas the a1 isoform was expressed in both cell types at about the same level. We also found that a3 expression was greater in osteoclasts with 10 or more nuclei as compared with osteoclasts with five or fewer nuclei. We hypothesize that these differences in a3 expression may be associated with previously demonstrated differences between large and small osteoclasts with reference to their resorptive activity.

Large osteoclasts predominate in diseases associated with accelerated bone loss. Bone is a dynamic tissue. To maintain its structural integrity, bone is continually remodeled, first being resorbed by osteoclasts and then remade by osteoblasts. An increase in osteoclastic activity relative to osteoblastic activity results in bone loss. Osteoclasts are multinucleated cells of hematopoietic origin. On average, osteoclasts in healthy bone compose a hydrophobic domain (V_o) spanning the membrane of the hydrophilic catalytic core (V_1), and the remaining four late other V-ATPase subunits to distinct cellular locations. isoforms of the V-ATPase “a” subunit may target or regulate other V-ATPase subunits to distinct cellular locations. V-ATPases are composed of at least 11 subunits; seven are part of the hydrophilic catalytic core (V_1), and the remaining four compose a hydrophobic domain (V_o) spanning the membrane bilayer. The hydrophobic domain contains one or two copies of the 100-kDa “a” subunit, the focus of this study. The mammalian “a” subunit has a hydrophilic ~48-kDa N terminus and a hydrophobic ~49-kDa C terminus containing up to nine putative transmembrane domains (13). In yeast, it has been shown that the “a” subunit is essential for V-ATPase assembly (14) and activity (15, 16) and interacts with V_1 subunits A and H (17). We have cloned two isoforms of the “a” subunit in yeast (Vph1p, and Stv1p (14, 18)) and have demonstrated differential localization of V-ATPase complexes containing either isoform.

Recent studies have shown that V-ATPases containing different “a” isoforms differ in their coupling efficiency and in vivo dissociation between the V_1 and V_o complexes (19). We hypothesize that isoforms of the “a” subunit may target or regulate other V-ATPase subunits to distinct cellular locations. The “a3” V-ATPase subunit may be essential for but not unique to the osteoclast plasma membrane V-ATPase.

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The nucletide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF393370, AF393371, and AF393372.

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The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST-S-RANKL, glutathione S-transferase-soluble receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase; RT, reverse transcriptase.
eukaryotes have four isoforms of the "a" subunit, all of which appear to have differential expression patterns (20–22). The a1, a2, and a3 isoforms have been shown to be expressed in most tissues examined. However, the a1 isoform is most highly expressed in brain and heart; a2 is most highly expressed in the acrosomal membrane within sperm (23) but also highly expressed in liver and kidney; and a3 is most highly expressed in liver and heart (24, 25). The a4 isoform appears to be kidney-specific (22, 26), and mutations within the human a4 isoform result in distal tubular renal acidosis (27, 28). Evidence suggesting that a3 is essential to osteoclastic function first came from its initial identification as "OC-116Kda." The OC-116Kda gene was cloned by a differential screening of a human osteoclastoma cDNA library, and its mRNA was reported to be uniquely expressed in multinucleated giant cells within osteoclastoma tumors (29). In mice, a3 was induced during osteoclast differentiation, and the gene product was localized in the plasma membrane and cytoplasmic filamentous structures within osteoclasts (24). Disruption of the mouse a3 encoding gene, (referred to as either AtP6i (30) or MUOC116 (31)) resulted in severe osteopetrosis, whereas mutations in the human a3 encoding gene (referred to as either TCIIRG1 (32) or OC116 (33)) resulted in a subset of autosomal recessive osteopetrosis (32) including infantile malignant osteopetrosis (33–35). Despite this compelling genetic evidence suggesting that a3 function is essential for and specific to osteoclastic bone resorption, expression studies suggest that a3 is ubiquitously expressed and probably also has roles unrelated to osteoclastic function. RT-PCR revealed that a3 was expressed in all tissues examined (36), whereas Northern blotting suggested that the highest a3 expression was in the liver (24, 25). Bone tissue was not investigated in either of these studies. One alternatively spliced transcript of a3, lacking the first 217 amino acids off the hydrophobic N terminus of a3, was identified as the T cell membrane protein TIRC7 and was suggested to have a central role in T cell activation in vitro and in vivo (37, 38).

To address the disparities conclusions drawn from the genetic evidence and the expression patterns of a3, we decided to directly compare a3 expression levels between bone and other tissues and the expression of a3 within the different bone cells. Finally, considering the differences between large and small osteoclasts (generally day 5) or within large osteoclasts (generally day 8). Prior to protein isolation, osteoclasts were further selected by gently washing the dishes with 3 × 10 ml of phosphate-buffered saline using a 10-ml pipette. To extract protein, 8 ml of TRIzol (Stratagene) was added per dish, and the isolation was performed according to the manufacturer's instructions.

**EXPERIMENTAL PROCEDURES**

**Materials—**The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC catalog no. TIB-71). Medium 199, α-minimum essential medium, Dulbecco's modified Eagle's medium, antibiotics (penicillin G, gentamicin, and fungizone) were obtained from Invitrogen. Sterile fetal bovine serum was obtained from MEDICORP Inc. Kits for mRNA purification and RT-PCR were purchased from Roche Applied Science. Polyclonal antibodies to the V-ATPase a1 and a3 subunits were a kind gift from Dr. Beth Lee (Washington University School of Medicine), and monoclonal antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Abcam. Most other reagents were from either Sigma or Fisher.

**Rabbit Osteoclast Isolation—**Osteoclasts were obtained from 10-day-old New Zealand White rabbits as described in Ref. 39. Briefly, the long bones (femurs, tibiae, humeri, and radii) were dissected out, adherent soft tissues were removed, and the cleaned sheets were cut longitudinally. The interior surfaces were then cutreated to release the bone cells, followed by repeated pipetting to release additional cells attached to the bone fragments. Cells were resuspended in α-minimum essential medium (pH 7.4) with 10% fetal calf serum and antibiotics (100 μg/ml penicillin G, 0.5 μg/ml gentamicin, and 0.2 μg/ml fungizone) and allowed to attach overnight to culture dishes in humidified air (37 °C and 5% CO₂). If further purification was required, cultures were incubated the following day with 0.001% protease E, 0.01% EDTA at room temperature for 10 min. Detached cells were washed away with culture medium, resulting in a 95% pure preparation of osteoclasts still attached to the culture dishes.

**Differentiation and Isolation of Large and Small Osteoclasts from the RAW264.7 Cell Line—**Approximately 5 × 10⁶ third passage RAW264.7 cells were cultured in a 100-cm² dish with 15 ml of Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 20 units/ml penicillin, 20 μg/ml streptomycin, 0.15 μg/ml fungizone, and 200 ng/ml recombinant glutathione S-transferase-soluble receptor activator of NF-κB ligand (GST-sRANKL) in humidified air (37 °C and 5% CO₂) with medium changes at days 3, 5, and 7. For protein isolation, 10 × 10⁶ cells were plated as described above and visually inspected until at least 78% of the total nuclei were contained within small (≤5 μm) osteoclasts (generally day 5) or within large osteoclasts (generally day 8). Prior to protein isolation, osteoclasts were further selected by gently washing the dishes with 3 × 10 ml of phosphate-buffered saline using a 10-ml pipette. To extract protein, 8 ml of TRIzol (Stratagene) was added per dish, and the isolation was performed according to the manufacturer's instructions.

**RT-PCR—**95% pure preparations of osteoclasts (prepared as described) were immediately suspended in lysis buffer (mRNA Capture Kit; Roche Applied Science) and stored at −80 °C until further use. Once mRNA was extracted using the mRNA capture kit, RT-PCR was immediately performed using the RT-PCR Titan One Tube system (Roche Applied Science). The reverse transcription was performed at 50 °C for 30 min followed by 35 cycles of PCR (30 s at 94 °C, 30 s at 55 °C, and 68 °C for 1 min).

**Oligonucleotides for RT-PCR—**To clone potential isoforms of the "a" subunit from rabbit osteoclasts, oligonucleotides were designed to encode four evolutionarily conserved regions within the C terminus of the 100-kDa V-ATPase subunit nucleic acid sequences obtained from rats, cows, humans. Oligonucleotides were made 2–5-fold redundant to account for base pair differences found between species. Restriction sites for XhoI and SalI were encoded into the 5′ ends of the sense and antisense primers, respectively, to facilitate subcloning into pBluescript PBS-SK (Stratagene). Sequences (with base pairs in parentheses indicating regions of redundancy) of the four primers are as follows: M061 (sense strand) 5′-ggcattgaattcctttatggagttgaggaa-3′; M062 (antisense strand), 5′-gatctgactctcttcgaagagctgagggaa-3′; M063 (sense strand), 5′-catgtagattccatcttcctgatctgaatgaa-3′; M064 (antisense strand), gcggatctgactctcttcgaagagctgagggaa-3′

**Plasmids, DNA Sequencing, and Sequence Analysis—**For a housekeeping gene, a 292-bp fragment (from position 289 to 581 according to GenBankTM accession number AF393370) encoding rabbit V-ATPase was used. The following plasmids were all obtained by performing RT-PCR with the indicated oligonucleotides on mRNA isolated from a 95% pure preparation of osteoclasts obtained from the long bones of New Zealand White rabbits. RT-PCR products were cut with XbaI and SalI (restriction sites added to the 5′ end of the oligonucleotides) and cloned into pBluescript pBS-SK. The inserts in the plasmids described below were all commercially sequenced (York University Core Molecular Biology and DNA Sequencing Facility, Toronto, Ontario) to completion on both strands using the T3, T7, or custom synthesized oligonucleotides. Sequences deposited in GenBank™ are the result of sequencing at least two independently obtained RT-PCR products. Sequence analysis was performed using the Wisconsin Package, version 10.0 (Genetics Computer Group (GCC), Madison, WI). The following plasmids were obtained. pRab-a1-1 (MM642; GenBank™ accession number AF393370) contains a 470-bp RT-PCR product resulting from oligonucleotides MO61 and MO62 (described above). It encodes the C terminus of the rabbit a1 isoform of the 100-kDa V-ATPase subunit. pRab-a1-2 (MM644; GenBank™ accession number AF393370) contains a 650-bp RT-PCR product resulting from oligonucleotides MO63 and MO64 (described above). It encodes the C terminus of the rabbit a1 isoform of the 100-kDa V-ATPase subunit, and its sequence is continuous with the 5′ end of pRab-a1-1. pRab-a2 (MM655; GenBank™ accession number AF393371) contains a 800-bp RT-PCR product obtained from oligonucleotides MO61 and MO63 (described above). It encodes the C terminus of the rabbit a2 isoform of the 100-kDa V-ATPase subunit.

**Plasmids**—pRab-a3 (MM656; GenBank™ accession number AF393372) contains a 950-bp RT-PCR product resulting from oligonucleotides MO61 and MO64 (described above). It encodes the C terminus of the rabbit a3 isoform of the 100-kDa V-ATPase subunit.

**Primers**—Reverse transcription was performed at 50 °C for 30 min followed by 35 cycles of PCR (30 s at 94 °C, 30 s at 55 °C, and 68 °C for 1 min).
exceptions, cDNA encoding a3 (pRab-a3) and β-actin were labeled using random hexamers and [32P]dCTP, with unincorporated nucleotides removed using an Amersham Biosciences NICK column. Prehybridization (68 °C for 2 h) and hybridization (68 °C for 3 h) were performed using Expresshyb hybridization solution (Clontech). After hybridization, the blots were washed three times for 15 min each in 2× SSC, 0.1% SDS at 60 °C followed by two times for 20 min each in 0.1% SSC, 0.1% SDS at 61 °C. The resulting autoradiograms were scanned using a transparency adapter, and band intensities were quantified using AlphaEase Image Analysis Software (Alpha Innotech Corp.).

Quantification of Immuno-blot—Immunoblots were performed exactly as described in Ref. 16, with the resulting signal obtained using the ECL detection system (Amersham Biosciences) and the FluorChem Imaging System (Alpha Innotech Corp.). To ensure that the chemiluminescent signals from the immunoblots were within the linear range, each protein sample was run on SDS-PAGE as a series of four serial dilutions. Multiple exposure times were recorded by the CCD camera, and an exposure time was used for quantification only if the obtained signal internally reflected the serial dilution of the sample. For each separate gel, the absolute value obtained for a1 and a3 at each of the four protein concentrations was divided by the absolute value obtained for GAPDH. GAPDH was used to normalize the signal between different samples, since exceptions. The a3 signal was first normalized to β-actin and then presented relative to a3 expression in bone.

RESULTS

Deletions and mutations within the mouse and human a3 genes result in an osteopetrotic phenotype (30–33) despite the fact that a3 expression has been reported in almost all tissues examined (36). To address these conflicting data, we set out to directly compare the expression level of a3 in osteoclasts with that in several other tissues. This study was initiated by first determining which of the four known isoforms of the 100-kDa “a” subunit are expressed in rabbit osteoclasts.

Bone Cells Express the a1, a2, and a3 Isoforms of the 100-kDa V-ATPase Subunit—To identify all V-ATPase isoforms expressed in osteoclasts, we employed an RT-PCR strategy and designed two sense and two antisense oligonucleotides, each encoding evolutionarily conserved regions found in all four of the a isoforms in several different species. These primers were used to perform several RT-PCRs on mRNA extracted from a 10-day-old rabbit femur as described under “Experimental Procedures.” RT-PCR products were pooled and cloned into sequencing vectors. Restriction mapping demonstrated that all 60 of the resulting recombinant plasmids fell into only three groups (data not shown). Sequencing revealed that we had cloned three isoforms of the 100 V-ATPase “a” subunit (sequencing data have been deposited in the GenBankTM/EMBL Data Bank with accession numbers AF393370, AF393371, and AF393372). These three rabbit isoforms have 91, 86, and 82% amino acid identity, respectively, to the a1, a2, and a3 identified in mice (24, 25) and 85, 65, and 68% amino acid identity, respectively, to the a1, a2, and a3 identified in chickens (43). The percentage similarity and the phylogenetic relationship among these isoforms suggests that we have cloned the ortholog a1, a2, and a3 subunits from rabbits and that all three isoforms are expressed in a 95% pure osteoclast preparation. Considering the numerous RT-PCRs performed and the number of clones sequenced, we next examined the expression of these isoforms in large and small bone-resorbing osteoclasts.
ber of recombinant plasmids screened, the fact that we did not retrieve the fourth isoform of the 100-kDa subunit supports the view that the a4 isoform is specific to kidney.

The a3 Isoform of the 100-kDa Subunit Is Highly but Not Exclusively Expressed in Osteoclasts—The pattern and magnitude of a3 expression is still controversial. The original studies using Northern blot analysis demonstrated that a3 was exclusively found in osteoclastomas and not expressed in kidney, liver, skeletal muscle, or brain (29). Subsequent studies using RT-PCR revealed, however, that a3 was expressed in all tested human tissues (36), and Northern analysis by two different groups demonstrated that the highest expression of a3 was in the liver (24, 25). In an attempt to understand why mutations and deletions of this apparently ubiquitously expressed gene result in an osteopetrotic phenotype, we compared the expression levels of a3 in brain, kidney, liver, lung, muscle, and spleen with that in bone. Northern analysis was performed using the a3 gene and, to account for differences in loading, β-actin. With the a3 probe, we found a single intense band at ~3.2 kb (data not shown). Quantification of the resulting autoradiographs (Fig. 1) shows that whereas bone does have the highest level of a3 expression, liver has only 10% less, with decreasing levels seen in kidney, brain, lung, spleen, and muscle. However, bone is a complex tissue, and osteoclasts represent at most 1% of the cells found within bone. If a3 mRNA expression were restricted to osteoclasts, this result would imply that the a3 subunit is highly expressed in osteoclasts. We therefore determined the expression of a3 in the various cell types within bone by per-
forming in situ hybridization (Fig. 2). Sequential bone slices were probed with a3 sense (right panel, negative control) and antisense (middle panel) RNA, and osteoclasts were identified by TRAP staining (left panel). Fig. 2 reveals that a3 expression within bone is mostly limited to osteoclasts.

To quantify the results shown in Fig. 2, in situ hybridization was performed on isolated osteoclast-containing cell populations using 3H-labeled a1 and a3 cDNA probes. Fig. 3A shows that the a3 isoform was highly expressed in multinucleated cells but barely detectable in mononuclear cells. For quantification, the number of grains per cell was divided by the number of nuclei per cell to normalize counts and thereby account for the variability in cell size. The results show that the a3 isoform is expressed almost 30-fold higher in multinucleated cells compared with mononuclear cells (Fig. 3B). In contrast, the a1 isoform appears to be expressed at low but equal levels in both multinucleated and mononuclear cells, whereas the housekeeping gene, GAPDH, is expressed significantly higher in the mononuclear cells. These results thus support the view that the a3 expression in bone reflects predominantly expression within osteoclasts.

The a3 Isoform Is Differentially Expressed in Large (≥10 Nuclei) and Small (≤5 Nuclei) Osteoclasts—Osteoclasts are heterogeneous, differing in size, shape, and resorptive activity. Large osteoclasts (defined as having ≥10 nuclei), are more likely to be found in diseases characterized by increased bone resorption (1–3), more likely to be in a resorptive state (4), and are more dependent on V-ATPases to recover from an acid load (5) than small osteoclasts (i.e., ≤5 nuclei). Considering the differences in V-ATPase activity between large and small osteoclasts, we addressed whether there was a concomitant difference in a3 expression, as suggested by visual inspection of the in situ hybridization shown in Fig. 4. As before, defining small as ≤5 nuclei and large as ≥10 nuclei is arbitrary and made to facilitate both the analysis of the data and comparison of the data with previous studies by creating two distinct nonoverlapping categories (4, 5). The number of nuclei per osteoclast, rather than surface area, was chosen to define cell size, since

![Image](https://example.com/image.png)
cytoplasmic volume is known to correlate with the number of nuclei. Surface area is an unreliable measure of size, because osteoclasts in a migratory phase are spread out, whereas in a stationary phase they assume a more rounded shape (44). Quantification of these results shows that whereas there is no difference in the expression of the housekeeping gene GAPDH, the expression of the a1 isoform in small osteoclasts is slightly but significantly higher than that in large osteoclasts. Conversely, a3 mRNA expression is 2.5-fold higher in large than in small osteoclasts (Fig. 4E). Results comparing osteoclasts containing 2–5, 6–9, and ≥10 nuclei suggest a gradual increase in a3 expression with an increase in osteoclast size (data not shown).

To test whether this observation was mirrored in a3 protein expression, it was necessary to switch from using authentic rabbit osteoclasts to osteoclasts differentiated from the mouse macrophage cell line RAW267.4 since insufficient numbers of osteoclasts can be isolated from rabbit long bones. RAW267.4 cells can differentiate into osteoclasts by culturing in media containing sRANKL. By varying the concentration of GST-sRANKL, the number of media changes, and the culture period, we obtained cultures in which at least 78% of the total nuclei within the culture dish were contained within small (≤5 nuclei) or large (≥10 nuclei) osteoclasts (Fig. 5). The number of osteoclasts with 6–9 nuclei was less than 5% for the two time points selected (data not shown). There were variations in the number of days required to achieve the results shown in Fig. 5 (plus or minus 1 day). Therefore, for all subsequent experiments, cultures were visually inspected on a daily basis, and protein was not extracted until enriched populations of large and small osteoclasts were obtained that were equal to or better than that shown in Fig. 5. Immunoblots of protein extracted from these two populations reveal that the level of a1 and a3 translation in large and small osteoclasts reflects the pattern of transcription found in authentic osteoclasts by in situ hybridization (Fig. 6). The a3 expression was 2.86 ± 0.79-fold (mean ± S.E., p < 0.05) more in large osteoclasts than in small osteoclasts, whereas a1 expression was similar (0.81 ± 0.33-fold; mean ± S.E., p > 0.05) in large and small osteoclasts.

These results lead us to speculate that the differences in a3 expression may be associated with the differences in the regulation of the intracellular pH (5) and resorptive activity (4) between large and small osteoclasts. Interestingly, ~4% of the small osteoclasts had a3 expression levels similar to large osteoclasts (>30 grains/nuclei). These numbers seem to correlate with the observation that 5.6% of small osteoclasts are actively resorbing (4), which could further suggest that a3 expression is associated with the resorptive potential.

**DISCUSSION**

An ideal target for an antiresorptive therapeutic would be a protein that is both uniquely expressed in osteoclasts and essential to osteoclastic function. Even more advantageous would be a therapeutic that could preferentially inhibit pathological osteoclast activity resulting in bone loss while not affecting osteoclasts engaged in maintaining bone integrity. The most commonly used class of antiresorptive therapeutics, bisphosphonates, are effective not because their targets are unique to osteoclasts or to osteoclast function but rather because bisphosphonates accumulate mostly in bone and are selectively taken up by resorbing osteoclasts (45). This nonspecificity results in other tissues being affected that are exposed to higher concentrations of bisphosphonates, such as the gastrointestinal tract, resulting in side effects such as esophageal ulcers and gastro-

![Fig. 5. Populations enriched in large or small osteoclasts can be obtained from RAW267.4 cells. A, RAW267.4 cells were cultured as described under “Experimental Procedures” with 200 ng/ml GST-sRANKL and media changes at days 3, 5, and 7. On days 5 (left panel) and 8 (right panel), plates were fixed and stained for TRAP. B, the number of cells and the number of nuclei in each cell were counted for the following groups: TRAP-negative mononuclear cells (white), TRAP-positive cells containing ≤5 nuclei (gray), and TRAP-positive cells containing ≥10 nuclei (black). To account for differences in cell size, the histogram (B) is presented as the percentage of total nuclei in each group.](http://www.jbc.org/Downloadedfrom)
intestinal infections (46, 47). The first reports describing the cloning and expression of the V-ATPase a3 subunit indicated that this isoform was both unique to osteoclasts and essential to osteoclast function (29), suggesting that a3 would make an excellent target for antiresorptive agents. Deletions and mutations within the mouse and human a3 gene result in an osteopetrotic phenotype (30–35), further supporting the notion that a3 is both unique and essential for osteoclasts and hence an excellent target for antiresorptive agents. However, expression studies show a ubiquitous distribution (24, 25, 36), suggesting that a3 would not be a suitable therapeutic target. To address why mutations in a ubiquitously distributed protein result in osteopetrosis and to examine whether a3 is indeed a suitable target for an antiresorptive therapeutic, we have directly compared a3 expression levels between bone and other tissues and compared the expression of a3 within the different bone cells.

Similar to previous results, we found high levels of a3 mRNA expression in liver with decreasing amounts in kidney, brain, lung, spleen, and muscle, but the highest expression was found in mRNA extracted from bone (Fig. 1). Furthermore, in situ hybridization demonstrated that within bone, a3 expression was mostly in osteoclasts (Fig. 2). Considering that osteoclasts represent less than 1% of bone cells, and assuming an even distribution of a3 in liver, these results would suggest that a3 expression in osteoclasts is ~100-fold higher than in liver cells. This might explain why mutations and deletions within the a3 gene result in an osteopetrotic phenotype. However, considering the high levels of a3 expression in liver and its ubiquitous distribution in other tissues, it is still surprising that other phenotypes do not result from a3 mutations. There is a precedent for osteoclasts being unique in this respect: Src –/– mice are also osteopetrotic due to deficient osteoclast function, whereas Src expression is also ubiquitous (46). A possible explanation may be that other isoforms can compensate for the lack of a3 in all tissues except osteoclasts. Further evidence that other isoforms may compensate for deficiencies, in particular for the “a” subunits, comes from mutational studies on the yeast ortholog genes.

Yeast have two isoforms of the V-ATPase “a” subunit, Vph1p and Stv1p (14, 18). In wild type cells, Vph1p is the predominantly expressed isoform and is localized to the yeast vacuole. Disruption of the VPH1 gene eliminates vacuolar acidification and results in a phenotype similar to but not as severe as disruptions of other V-ATPase subunits encoded by single genes. This decrease in severity of the phenotype is most likely due to the presence of the second isoform, Stv1p. Overexpression of STV1 in Δvph1 strains results in mislocalization of Stv1p to the yeast vacuole, restores vacuolar acidification, and eliminates all Δvph1 phenotypes, demonstrating that Stv1p can functionally complement Vph1p. Disruption of STV1 does not result in any detectable phenotype, presumably because the constitutional high expression of Vph1p enables it to functionally complement Stv1p absence.

If a similar mechanism were operating in osteoclasts, deletions or mutations of one of the four mammalian V-ATPase “a” subunits could be partially compensated by the remaining three isoforms. A phenotype might only arise when very high expression levels of one particular isoform are required for a specific function. Considering the ubiquitous distribution of a3 within mammalian cells, it is likely that this isoform is responsible for acidifying other compartments such as the lysosome (47). Deletion of the a3 gene might not necessarily affect the acidification of other organelles if the other isoforms are able to functionally compensate for its absence. Considering that we have shown that the expression levels of a1 and a3 are similar in mononuclear cells (Fig. 3), it would seem possible that an a3 deficiency is compensated in tissues where other isoforms are normally expressed to a similar degree as a3. Since a3 expres-
sion appears to be much higher than that of a1 in multinecled osteoclasts (Fig. 3), the levels of a1 (and possibly a2) may not be sufficient to compensate for the absence of a3, hence the osteopetrotic phenotype that results from mutations within a3.

Osteoclast size has been shown to increase in diseases characterized by increased bone resorption such as end stage renal disease (2), Paget’s disease (48), periodontal disease (3, 49), and rheumatoid arthritis (50). We have previously shown that large osteoclasts, as a population, are more active resorbers than small osteoclasts and that the reason for this is that the proportion of large osteoclasts that are in a resorptive state is larger (40%) than that of small osteoclasts (5.6%) (4). Here we show that a3 mRNA (Fig. 4) and protein (Fig. 6) expression are higher in large compared with small osteoclasts, suggesting that a3 expression may be associated with the differences in the resorptive activity between large and small osteoclasts. We also observed that ~4% of small osteoclasts had a3 expression levels similar to large osteoclasts. This correlates well with the observation that 5.6% of small osteoclasts are actively resorbing bone.

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REFERENCES
1. Singer, F. R., and Rodman, G. D. (1996) in Principles of Bone Biology (Bilezikian, J. P., Raisz, L. G., and Rodan, G. A., eds) pp. 969–977, Academic Press, Inc., San Diego, CA
2. Kaye, M., Zucker, S. W., Leclerc, Y. G., Prichard, S., Hodsman, A. B., and Kay, W. R. (1996) in Bone (Henderson, J. E., and Goltzman, D. G., eds) pp. 363–368, Humana Press, Clifton, NJ
3. Singer, F. R., and Roodman, G. D. (1996) in The Osteoporosis Primer (Bilezikian, J. P., Keeling, D. J., Andersson, A. K., Wallbrandt, P., Zecca, L., et al.) pp. 969–977, Humana Press, Clifton, NJ
4. Lees, R. L., and Heersche, J. N. (2000) J. Bone Miner. Res. 15, 1164–1171
5. Heinemann, T., Bulwin, G. C., Randall, J., Schnieders, B., Sandhoff, K., Volk, H. D., Milford, E. L., and Gullans, S. R. (1998) Immunity 9, 599–618
6. Manolson, M. F., and Heersche, J. N. M. (2000) in Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
7. Komminoth, P. (1996) in Nonradioactive In Situ Hybridization Application (Grunewald-Janho, S., Keesey, J., Leous, M., van Miltenburg, R., et al.) pp. 137–146, Springer-Verlag, New York.
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