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Molecular Cloning and Characterization of a Putative Novel Human Osteoclast-Specific 116-kDa Vacuolar Proton Pump Subunit

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A cDNA encoding a possible novel human 116-kDa polypeptide subunit of the osteoclastic proton pump (OC-116KDa) has been identified by differential screening of a human osteoclastoma cDNA library. The predicted sequence of OC-116KDa consists of 822 amino acids and is 46.9% and 47.2% identical at the amino acid level to the 116-KDa polypeptide of the vacuolar proton pump of rat and bovine brain respectively. OC-116KDa mRNA was found at high levels in osteoclastomas by Northern analysis but was not detected in tumor stromal cells or in other tissues including kidney, liver, skeletal muscle and brain. OC-116KDa mRNA was localized to multinucleated giant cells within the osteoclastoma tumor by *in situ* hybridization.

Osteoclasts, multinucleated giant cells which are responsible for bone resorption, degrade both the inorganic and organic components of bone in a local area subjacent to the matrix attachment site (1). Dissolution of the hydroxyapatite mineral phase is dependent upon acidification of the subosteoclastic resorption lacuna, via the action of carbonic anhydrase II and a proton pump (2,3,4). Immunological cross-reactivity with antibodies to the endosome and coated vesicle proton pumps have indicated that the osteoclast proton pump is related to V-type proton pumps (5,6).

V-type proton pumps are multi-subunit complexes with two distinct functional domains: a peripherally-associated cytoplasmic catalytic sector that contains 70-(subunit A), 58-(subunit B), 40- and 33-kDa subunits (7); and a proton channel, which is likely composed of the 116-, 39-, and 17-kDa components (8). V-type proton pumps have wide distribution and are evolutionarily conserved. Thus the A and B subunits have greater than 75% conservation of primary structure extending from Archaebacteria to man (9–17). The rat and bovine 116KDa subunits have greater than 96.6% conservation of primary structure (18,19).

In eukaryotic cells, V-type proton pumps are located to most intracellular organelles of both constitutive and specialized secretory pathways. There are two possibilities for proton pump subunit diversification: first, that there are organelle- or cell-specific isoforms of the subunits; or second, that there are organelle- or cell-specific family member genes of the proton pump subunits. In support of the first possibility, two isoforms of the integral 116-kDa (19) and 17-KDa (22) subunits, as well as of A (23), B (24,25) and E (26) subunits have been reported, generated by alternative splicing. However family member genes of the proton pump subunits have not yet been described. It is also unknown if osteoclast-specific proton pump subunits exist.

In this study, we report the isolation of a gene encoding a possible human 116-kDa polypeptide of the vacuolar proton pump, which appears to be uniquely expressed in osteoclastic cells.

MATERIALS AND METHODS

Cells and cell culture. Human osteoclastoma tumors were obtained courtesy of Dr. Andrew Rosenberg. Osteoclastomas consist of ~30% multinucleated tartrate resistant acid phosphatase positive (TRAP+) giant cells. These cells possess a closely similar phenotype to osteoclasts and are also capable of excavating resorption pits on bone slices (27,28). The stromal cells from the tumors were obtained as described in (29). Osteoblastic (HOS-TE85), myelomonocytic (U-937), T lymphocyte (HSB-2), neuroblastoma (SK-N-MC), pancreatic adenocarcinoma (AsPC-1) and normal skin fibroblast (CRL 1467) cell lines were purchased from ATCC, Bethesda, MD. The epithelial cell line Hep-2 was kindly provided by Dr. Margaret Duncan. Normal rat osteoblasts (ROB) were obtained as described in (36).

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Library construction and differential screening. Two human osteoclastoma cDNA libraries were prepared in pcDNAII vector (InVitrogen) and in the Lambda-ZAP system (Stratagene) (29). Differential screening was performed as described in (29). Briefly, clones were randomly picked from the pcDNAII library and were hand plated in triplicate on nitrocellulose filters. Mixed cDNA probes were produced from mRNA isolated from the osteoclastoma tumor and from propagated stromal cells. The clones which were reactive with the tumor probe, but were unreactive or only weakly reactive with the stromal cell probe were isolated. Purified DNA from these clones were rescreened in a dot blot format to confirm the original result.

cDNA cloning and sequencing. For full length cDNA characterization, A 1.0 kb putative proton pump probe labelled with α^{32}PdCTP was used to screen the Lambda-ZAP osteoclastoma library. Positive clones were purified, and the size of inserts was determined following excision with Kpn1 and Xba1. A clone containing a full-length insert of 2.6 kb was subjected to controlled digestion with ExoIII to generate a series of diminishing insert sizes. Sequence analysis was then carried out from both ends by the dideoxy method (30) using the Sequenase kit (U.S. Biochemical Corp.). Homologies were compared with known proton pump sequences using the BLAST program at N.C.B.I.

Northern blotting. Total RNA from osteoclastomas and cell lines was isolated by the method of Chomczynski and Sacchi (31). Whole cell RNA from human tissues was purchased from Clontech, Palo Alto, CA. Total cellular RNA was separated on a 1.0% agarose gel containing 6% formamide and transferred to nylon membranes. The integrity and quality of RNA was confirmed by ethidium bromide staining. 1.0 kb 3′ end of OC-116KDa cDNAs were used as probes. Probes were radiolabeled with α^{32}pdCTP using a random primer labeling kit (Stratagene). Hybridization was performed as described previously (29).

In situ hybridization. In situ hybridization was performed as described in (29).

RESULTS

Differential screening. Approximately 12×10^3 clones from the pcDNAII osteoclastoma library were replica-plated and were screened by differential hybridization as described previously (29). One clone contained 1.0 kb insert which gave a positive hybridization signal with tumor cDNA, but was negative with stromal cell cDNA, was found to possess approximately 50% homology to the rat 116KDa vacuolar type proton pump subunit, but was not identical to any known proton pump subunits. This sequence was designated OC-116KDa.

Northern analysis. Northern analysis of mRNA from the osteoclastoma tumor using the α^{32}P-labelled 1.0 kb 3′ OC-116KDa cDNA probe revealed a transcript of approximately 2.7 kb (Fig. 1). OC-116KDa mRNA was found at high levels in the osteoclastoma tumor, and at much lower levels in the human pancreatic adenocarcinoma cell line (AsPC-1), but was not detected in skeletal muscle, liver, kidney, or brain. OC-116KDa mRNA was also absent from osteoclastoma stromal cells, normal rat osteoblasts (ROB), as well as a panel of human cell lines: osteoblastic (HOS-TE85), myelomonocytic (U-937), T lymphocyte (HSB-2), epithelial (laryngeal carcinoma, HEp-2), neuroblastoma (SK-N-MC), and normal skin fibroblasts (CRL 1467).

cDNA cloning and sequence analysis of OC-116KDa cDNA. Rescreening the pcDNAII library failed to yield clones containing full-length inserts. A second library was therefore constructed in phage using the Lambda-ZAP system (Stratagene). Screening of this library yielded 25 positive clones, of which the two longest (p-18, p-43) contained inserts >2.6 kb. Complete bidirectional sequence analysis was carried on the p-43 clone. Four other clones including p-18 were partially sequenced. All sequences were identical.

The nucleotide and the deduced amino acid sequences of the OC-116KDa cDNA clone are shown in Fig. 2. The nucleotide sequence of the cDNA encoding the 116-KDa proton pump polypeptide contains 2622 bp excluding the 3′-poly(A) tail. The cDNA contains a 57 bp 5′-untranslated region, and a rather short 3′-untranslated region of 99 bp. The nucleotide sequence contains an open reading frame, starting from the first ATG codon, encoding an 822-amino acid polypeptide. At the 3′ end, the AATAAA sequence (Fig. 2, underlined) is a common polyadenylation signal. The cDNA is full-length as judged by the fact that its size corresponds well to the message size observed on RNA blots and that it contains an in-frame termination codon 5′ to the initiator methionine (underlined in Fig. 2). Database searches revealed that OC-116KDa shows 46.9% and 47.2 homology at the amino acid level with the classical 116KDa subunit of the vacuolar proton pump of rat and bovine respectively (18,19) (Fig. 3).

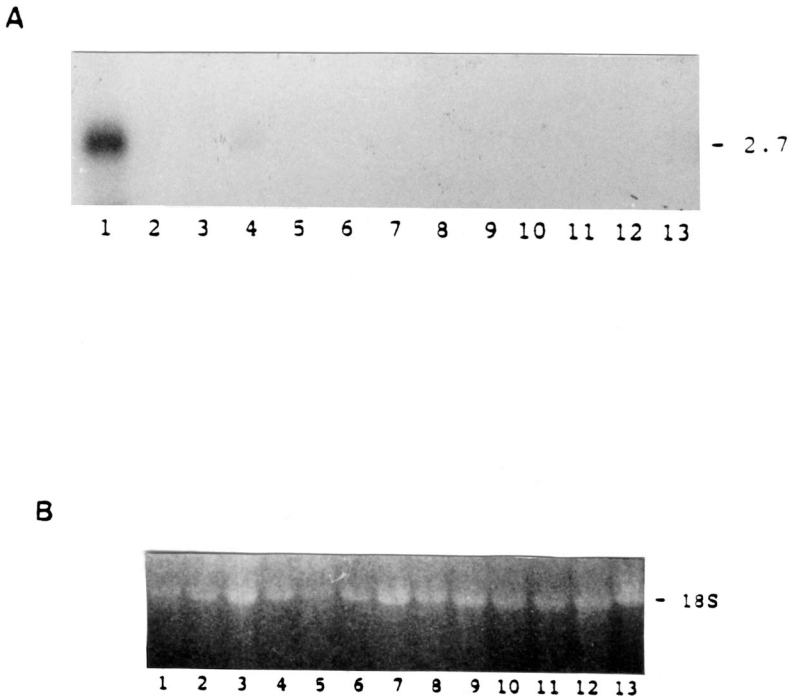

FIG. 1. Northern hybridization of OC-116KDa cDNA to total cell RNA from human tissues and cell lines. Lane 1, osteoclastoma tumor; lane 2, normal rat osteoblasts (ROB); lane 3, SK-N-MC; lane 4, AsPC-1; lane 5, U-937; lane 6, HOS-TE85; lane 7, Hep-2; lane 8, HSB-2; lane 9, skeletal muscle; lane 10, liver; lane 11, kidney; lane 12, brain; lane 13, stromal cells. (A) Autoradiography; (B) ethidium bromide stained gel.

The composition of OC-116KDa is characterized by an abundance of hydrophilic resides in the first 390 amino acids and a rather hydrophobic region in the following 432 amino acids. Hydrophobicity plots (33) indicate that at least six transmembrane regions are present in the carboxyl-terminal portion of the molecule. The putative transmembrane regions are separated by spacer regions of different length and hydrophilicity (Fig. 4).

Based on the hydropathy plots, OC-116KDa shows structural homology with other 116KDa hydrophobic membrane proteins with transport-related function, including rat- and bovine-116KDa (18) (Fig. 4). All three of these proteins are about 830 amino acids in length and contain six transmembrane domains with a hydrophilic region between domains (Fig. 4).

In situ hybridization. Cells within the osteoclastoma tumor which produce mRNA for OC-116KDa were identified by *in situ* hybridization. As shown in Fig. 5, a digoxygenin-labelled antisense probe was strongly reactive with all multinucleated osteoclasts, but was unreactive with stromal cells. In contrast, the sense probe produced only minimal background staining, which was not localized to any cell type.

DISCUSSION

In order to solubilize bone mineral and degrade the organic matrix of bone, osteoclasts must secrete 1–2 protons for every Ca^{2+} liberated. This transport is a major metabolic activity of osteoclasts and requires an electrogenic proton pump. The osteoclast proton pump possesses several unique features: a unique pharmacological profile, that is, the proton pump is osteoclast-derived membranes was not only shown to be sensitive to NEM and Bafilomycin A1, similar to the classical vacuolar proton pump, but also to vanadate, an inhibitor of P-type ATPase (34). Furthermore, the osteoclast-proton pump is the most active of all acid transport systems studied. Rat

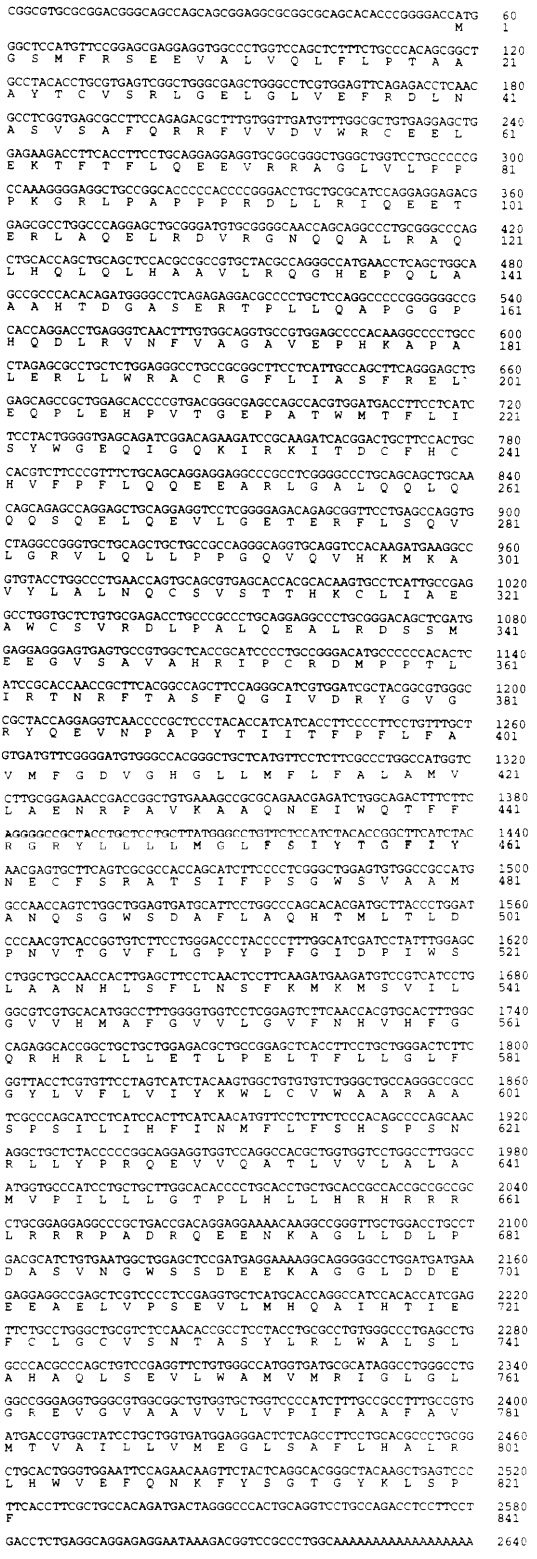

FIG. 2. Nucleotide and deduced amino acid sequences of human OC116-KDa. Numbers indicate the nucleotide bases.

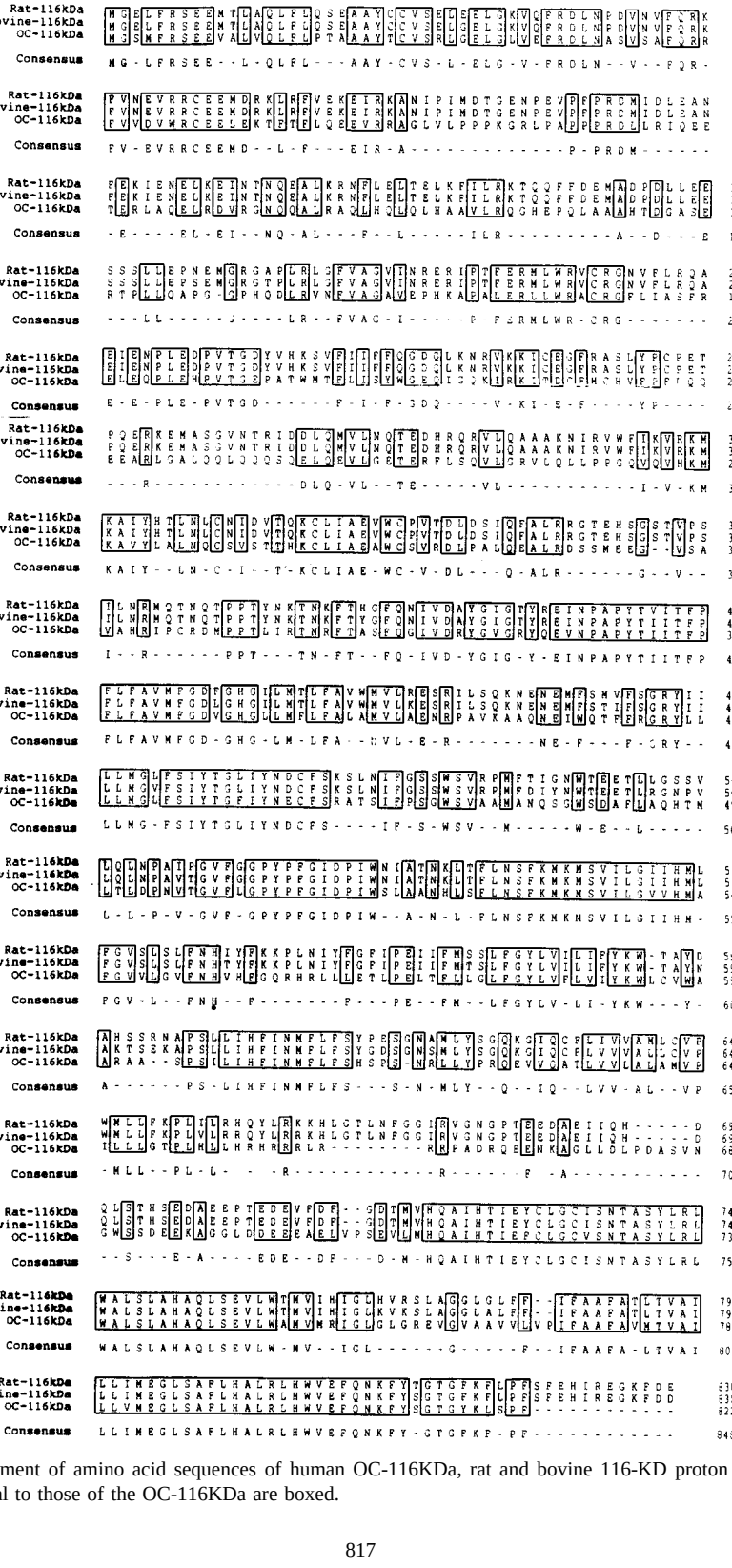

FIG. 3. Alignment of amino acid sequences of human OC-116KDa, rat and bovine 116-KD proton pump subunits. Residues identical to those of the OC-116KDa are boxed.

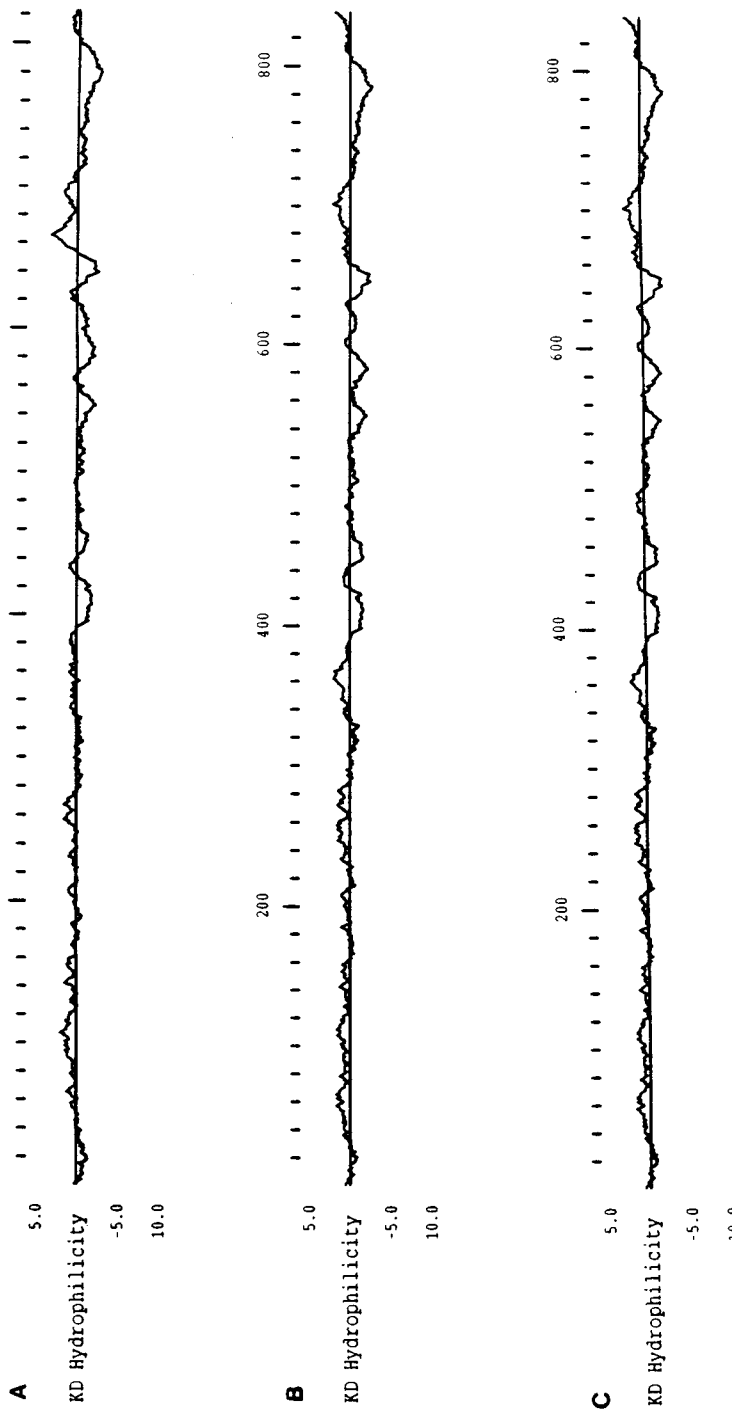

FIG. 4. Hydrophilicity plot of OC-116KDa protein (A) comparison with the rat (B) and bovine (C) 116-KD proton pump subunit.

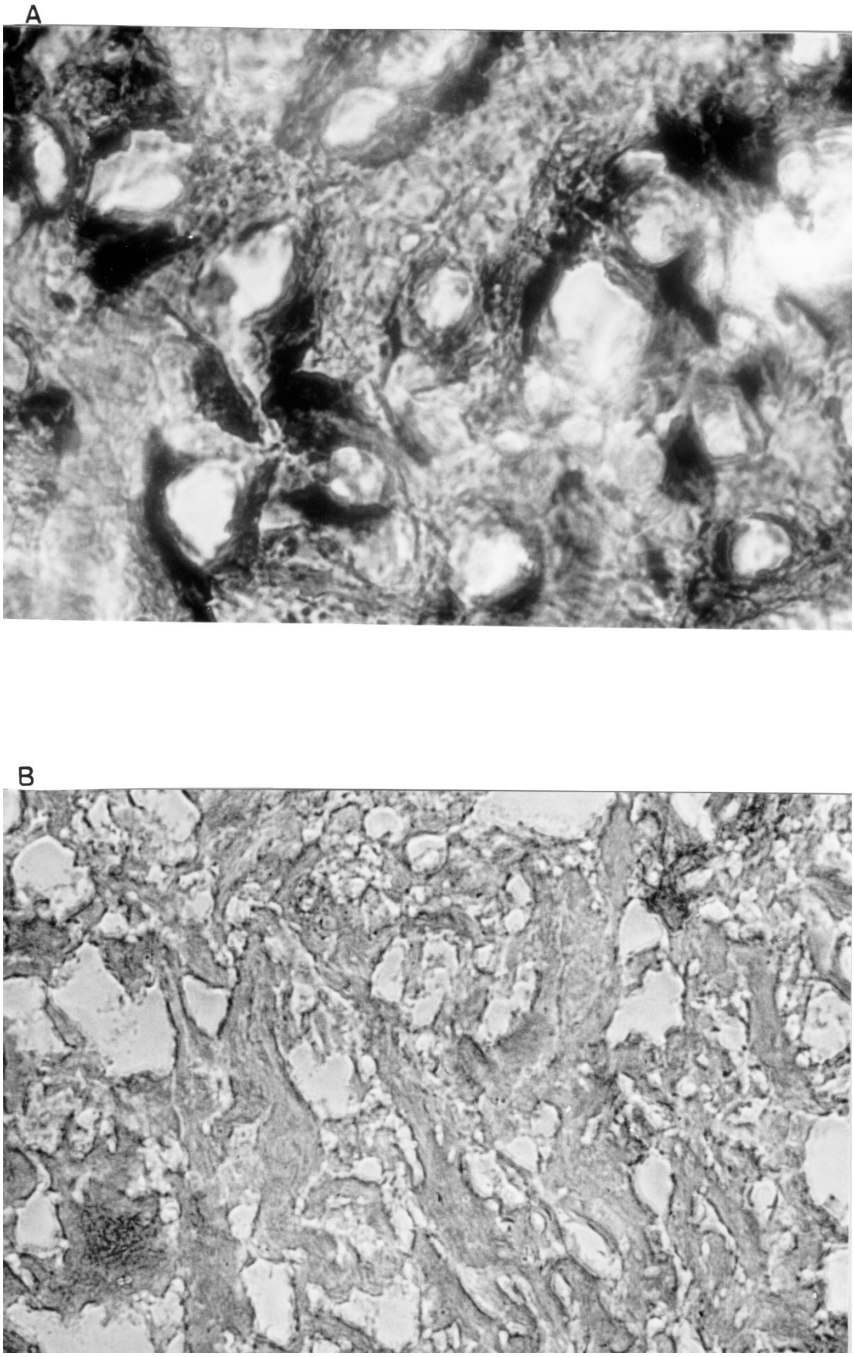

FIG. 5. *In situ* hybridization of OC-116KDa mRNA in a human osteoclastoma. cRNA probes were digoxygenin-labelled and were developed with an alkaline phosphatase-labelled antibody. Counterstain: methyl green. (A) Antisense; (B) sense control. Original magnification: ×400.

116KDa is 96.7% similar to bovine 116KDa at the amino acid level, whereas OC-116KDa had only about 47% homology to either rat- or bovine-116KDa. This fact suggest that OC-116KDa is the product of a separate gene rather than an alternatively splicing products. OC-116KDa mRNA was found at high levels in the osteoclastoma tumor but was not detected in other normal human tissues

(Fig. 1). This is in contrast to the ubiquitous distribution of the rat and bovine 116KDa subunit. The differences between OC-116KDa and classical 116KDa subunit of vacuolar proton pump may constitute part of the molecular basis for the precise regulation of expression of the osteoclast proton pump during the bone remodeling process. Taken together, these data support the hypothesis that OC-116KDa may represents a novel 116KDa subunit of proton pump which is distinct from the previously-described 116KDa subunit. Whether the OC-116KDa subunit plays an important role in the special properties of the osteoclast proton pump remains to be explored. The cell-specific expression of OC116-KDa might be useful as a target for therapeutic intervention in diseases with increased resorption of bone or cartilage, such as osteoporosis and osteoarthritis.

Although the function of the 116KDa subunit in the V-type proton pump is not definitively established, it appears to be an essential component of the vertebrate pumps (37), and is also present in lower unicellular eukaryotes and plants (32,38). In yeast, disruption by mutation of the gene encoding this subunit results conditional lethality (20,21). The 17- and 116KDa subunits are the components of the proton pump that are most hydrophobic (35). Based on hydrophilicity plots of the amino acid sequence, OC-116KDa shows structural homology with other 116KDa proton pump subunits (Fig 5) and also contains a large and highly charged amino-terminal domain of unknown function that may interact with the cytoplasmic catalytical sector. These data suggest that the OC- 116KDa subunit may be part of the proton-conducting, intramembranous complex of the vacuolar proton pump, and may also play a role in mediating the coupling between ATP hydrolysis by the cytoplasmic 70- and 58-kDa subunits, and proton translocation by the intramembranous subunits, including perhaps its own transmembrane regions (18). Further functional studies are needed to resolve these questions.

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