Three Subunit \(a\) Isoforms of Mouse Vacuolar \(H^+\)-ATPase

PREFERENTIAL EXPRESSION OF THE \(a3\) ISOFORM DURING OSTEOCLAST DIFFERENTIATION

(Received for publication, November 29, 1999, and in revised form, December 16, 1999)

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Vacuolar \(H^+\)-ATPase (V-ATPase) is a multi-subunit enzyme with a membrane peripheral catalytic \((V_1)\) and an intrinsic \((V_o)\) sector. We have identified three cDNA clones coding for isoforms of mouse \(V_o\) subunit \(a\) (\(a1\), \(a2\), and \(a3\)). They exhibit 48–52% identity with each other and high similarity to subunit \(a\) of other species. The \(a1\) isoform was mainly expressed in brain and liver. The \(a2\) isoform was observed in heart and kidney in addition to brain and liver. Transcripts for the \(a3\) isoform were strongly expressed in heart and liver. The \(a3\) isoform was constitutively expressed and localized in the cytoplasmic endomembrane compartments of the same cells. These findings suggest that the \(a3\) isoform is a component of the plasma membrane V-ATPase essential for bone resorption.

The acidification of intracellular compartments is driven by a vacuolar-type \(H^+\)-ATPase (V-ATPase) and is essential for processes such as zymogen activation, protein sorting, receptor-mediated endocytosis, and neurotransmitter uptake into synaptic vesicles (1–4). V-ATPases are also localized in the plasma membranes of epithelial cells of tissues such as kidney, seminal duct, and bladder (5–7). V-ATPase localized in the ruffled border membranes of osteoclasts secretes protons to dissolve mineral components of bones for remodeling (8, 9). A vacuolar-type \(H^+\)-ATPase (V-ATPase) 1 and is essential for bone resorption. These findings suggest that \(a3\) is a key subunit of V-ATPase for its localization in the plasma membranes of osteoclasts, and thus for bone resorption.

EXPERIMENTAL PROCEDURES

Materials—A Superscript™ mouse 13.5 day embryonic cDNA library (Superscript™) was prepared and sequenced using a model 377 DNA sequencer. The ddY male mice were purchased from Life Technologies, Inc. Radioactive materials were from Amersham Pharmacia Biotech. Enzymes for molecular cloning were from Takara Shuzo Co., Ltd. 1a,25-Dihydroxy cholecalciferol (1,25(OH)\(_2\)D\(_3\)) was from Sigma. The mouse EST clones were from Genome Inc. and American Type Culture Collection.

Cloning of Subunit \(a\) Isoforms—One mouse EST clone (AA444415) was prepared and sequenced using a model 377 DNA sequencer. The cDNA library was screened to isolate a full-length clone. By screening (1 \(\times\) 10\(^5\) colonies), a single clone was isolated and sequenced. The clone contained a full-length cDNA, although two introns (79 and 308 bp) were present in the open reading frame. To remove these introns, a DNA fragment was amplified by reverse transcribed PCR using mRNA prepared from 13.5-day mouse embryos, and then cloned. The resulting product was introduced into the corresponding region of the cDNA clone to construct a complete cDNA coding for the \(a3\) isoform.

Mouse EST clones (AA980626 and AA611922) coding for the \(a1\) and \(a2\) isoforms, respectively, were sequenced. The 5’ region of each clone was obtained by 5’-RACE using mRNA from 13.5-day mouse embryos. The PCR products were sequenced and ligated with the corresponding cDNAs to create full-length clones for \(a1\) and \(a2\). The entire cDNAs for \(a1\), \(a2\), and \(a3\) were 3815, 2917, and 2587 bp (not including polyadenylation), respectively.

Northern Blot—Northern blot analysis was carried out using multi-tissue blots (CLONTECH) according to the manufacturer’s recom
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RESULTS

Identification of Three Mouse cDNA Clones for V-ATPase Subunit a Isoforms—A search of mouse EST data bases using the amino acid sequence of bovine subunit a (30) led to the identification of three clones (AA980626, AA611922, and AA444415) that could code partial sequences of proteins similar to the subunit a of other species. Since one EST clone (AA444415) lacked the 5′ region, we searched a mouse cDNA library and obtained one full-length clone corresponding to the EST. For the other two clones (AA980626 and AA611922), 5′-RACE was carried out using 13.5-day embryo mRNA, and full-length cDNAs were constructed from the PCR products and the corresponding clones. Based on the similarity with the subunit a of other species (see below), the proteins encoded by the cDNAs were named the a1, a2, and a3 subunits (Fig. 1).

Amino Acid Sequences and Transmembrane Structures of Subunit a Isoforms—The a1, a2, and a3 isoforms comprised 832, 856, and 834 amino acid residues, respectively. Hydrophathy plot analysis (38) indicated that these isoforms had closely similar structures with nine putative transmembrane regions (Fig. 1, I–IX), as proposed by Leng et al. (39). Higher conservations were observed in the putative transmembrane regions. The a1 and a2 isoforms had single potential N-linked glycosylation sites at positions 489 and 505, respectively, whereas the a3 isoform contained three sites at positions 488, 504, and 595.

Western Blot—Cells were solubilized in 50 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol and 2% SDS, and then heated at 60 °C for 5 min. Samples were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Immunodetection was carried out using alkaline phosphatase-conjugated antibodies. The antibodies were purified using a recombinant protein (Macia Biotech). Anti-a antiserum was generated by immunizing a rabbit with a synthetic recombinant antigen (HRHRRNTQRRPAGQQDE, positions 660–676) that could code partial sequences of proteins similar to the subunit a of other species. Since one EST clone (AA444415) lacked the 5′ region, we searched a mouse cDNA library and obtained one full-length clone corresponding to the EST. For the other two clones (AA980626 and AA611922), 5′-RACE was carried out using 13.5-day embryo mRNA, and full-length cDNAs were constructed from the PCR products and the corresponding clones. Based on the similarity with the subunit a of other species (see below), the proteins encoded by the cDNAs were named the a1, a2, and a3 subunits (Fig. 1).
tical to a putative immunoregulatory protein from mouse T cells (25) except for a replacement (Ser → Cys) at position 486 and a Leu insertion at position 788 in the a2 isoform. This difference may be due to the mouse strains used for cloning. The a2 isoform exhibited 91% identity with the subunit α purified from bovine lung (34). The a3 isoform showed 84% identity with the polypeptide encoded by OC-116-kDa from a human osteoclastoma (31).

**Tissue Distributions of Subunit α Isoforms**—Northern blot analysis revealed that a 4.2-kb α1 transcript was present in all tissues examined (Fig. 2). High levels of expression were observed in brain and liver, as previously reported (30, 40). Expression of the α2 transcript was detectable in heart, brain, liver, and kidney. The α3 transcript was observed predominantly in heart and liver. Low but significant expression of the α3 isoform was found in brain, spleen, lung, and kidney. Transcripts of different sizes, possibly due to alternative splicing, were detected for the α2 and α3 isoforms: α2, 2.6, 3.0, and 4.9 kb; α3, 2.5 and 3.2 kb. We identified an EST clone (AA591593) for the α2 isoform, containing an additional 599-bp 3′ untranslated region, presumably corresponding to the splicing variant of the EST clone described above.

Southern blot analysis of mouse genomic DNA indicated that a single gene for the α3 isoform was present in the mouse genome (Fig. 3). The genomic DNAs for the α1 and α2 isoforms gave more than one band depending on the endonucleases used, suggesting that there may be related sequences in the genome.

**Induction of the α3 Isoform during Osteoclast Differentiation**—In osteoclasts, V-ATPase is distributed in the ruffled border membrane and acidifies the outside for bone resorption (8, 9). Thus, it became of interest to determine which isoform(s) was expressed in the plasma membrane of osteoclasts. Mouse bone marrow cells were cultured in the presence of 1,25(OH)₂ D₃ to develop osteoclasts in vitro (36). As shown in Fig. 4A, the α3 isoform was strongly expressed in the presence of 1,25(OH)₂ D₃. In contrast to α3, the α1 and α2 isoforms were constitutively synthesized regardless of the presence of 1,25(OH)₂ D₃; low but significant signals were observed for both isoforms. The time course of the appearance of the α3 isoform expression was consistent with the profile of osteocalcin differentiation (Fig. 4B). These results indicated that only the α3 isoform was induced during osteoclast differentiation. Consistent with the increase in the α3 isoform in the V₅ sector, the A and B subunits in the V₅ sector were also induced on the addition of 1,25(OH)₂ D₃ (Fig. 4A), suggesting that the entire V-ATPase was induced during osteoclast differentiation.

**Presence of the α3 Isoform in the Osteoclast Plasma Membrane**—V-ATPases related to bone resorption should be localized in the plasma membrane to acidify the resorption lacuna. The intracellular distributions of the α1, α2, and α3 isoforms were determined by immunofluorescence microscopy using affinity-purified antibodies. The α1 isoform was found in the dot-like structures dispersed throughout the cytoplasm (Fig. 5, A and B), whereas the signal of the α3 isoform was detected on the plasma membrane and its vicinity (Fig. 5, C and D). No signals were detected with the anti-α2 antibodies, probably due to the low level of α2 expression in osteoclasts (Fig. 4A). Consistent with the induction of subunit A and B of V₅ sector in the presence of 1,25(OH)₂ D₃, they were also detectable in the osteoclast plasma membrane (data not shown). These results indicated that the α3 isoform was predominantly located in the plasma membrane, and thus is a component of the V-ATPase responsible for bone resorption in osteoclasts.

Lee et al. recently reported that V-ATPase subunit E is co-localized with actin filaments in osteoclasts (13). The α3 isoform was also observed on the filamentous structures extending from the plasma membrane (Fig. 6, A and D). It was of interest to identify the filamentous structures. Actin filaments along the ruffled membrane were observed, as reported previously (10, 11). However, the filamentous structures stained with antibodies against the α3 isoform were different from actin filaments (Fig. 6, A–C). In contrast to actin filaments, microtubules were co-localized significantly with the filamentous structures (Fig. 6, D–F). These results indicated that the α3 isoform was associated with microtubules in osteoclasts.

**DISCUSSION**

We have identified three cDNA clones coding for the α1, α2, and α3 isoforms of mouse V-ATPase subunit α. They were expressed differently in the mouse tissues examined. The α1 isoform was mainly expressed in brain and exhibited high identity with subunit α from clathrin-coated vesicles of rat and bovine brain (30, 40). The α2 isoform exhibited 91% identity to the subunit α purified from bovine lung (34), although no α2 expression was detectable in mouse lung on Northern blotting. High levels of α3 expression were observed in brain and liver.
It is tempting to assume that the different expression patterns of the isoforms are responsible for the diverse functions of V-ATPases in these tissues. The a1 and a2 isoforms contained one putative N-linked glycosylation site, whereas a3 had three sites. Consistent with these numbers of sites, the a3 isofrom from mouse tissues migrated slower than the a1 and a2 ones on polyacrylamide gel electrophoresis.2

Yeast cells have two subunit a isoforms (Vph1p and Stv1p) (15, 16). Vph1p, the major form of subunit a, is localized in the vacuolar membrane, whereas Stv1p is located in the Golgi or endosomal membrane (16), suggesting that subunit a plays a functional role in targeting V-ATPases to their resident compartments. To determine which mouse isofrom corresponds to Vph1p or Stv1p, expression plasmid carrying cDNA for the mouse a1, a2, or a3 isofrom was introduced into the vph1 and stv1 mutant cells. However, no clones could complement the vph1 and stv1 mutations. These results are not surprising because the amino acid identities between Vph1p (or Stv1p) and mouse a isoforms are not high (36–40%).

Isoforms of V-ATPase subunit A, B, G, H, and a have been identified in vertebrates (25–27, 29–34). Mouse a subunits are the first example of all three isoforms being identified in the same higher eukaryote. Northern blot analysis revealed three transcripts for the a2 subunit and two for a3, possibly due to alternative splicing. These transcripts may cause further variation of subunit a. This variety of isoform combinations is enough to create the diversity of V-ATPases, which may establish different pH in acidic compartments. The different subunits may also contribute to the subcellular localization of the entire enzyme.

In osteoclasts, the B2 isoform is known to be specifically expressed and distributed in the plasma membrane (41, 42). Furthermore, the isoform was reported to be induced in osteoclasts generated from human blood monocytes (43). The a1, a2, and a3 isoforms were synthesized in osteoclasts, but only a3 was induced specifically and localized in the plasma membrane, suggesting that the V-ATPase subtype having the B2 and a3 isoforms is an inducible complex involved in bone reabsorption of osteoclasts. It is of interest to study the intracellular locations of the a3 isoform in heart and liver, where the a3 transcripts are predominantly expressed. The a2 isoform was expressed in thymus (25), implying that this isoform may be a component of the V-ATPase subtype related to immune responses.

Lee et al. (13) recently showed that subunit E of V-ATPase is co-localized and associated with actin filaments, suggesting that V-ATPase interacts with actin filaments during osteoclast activation. However, immunofluorescence analysis revealed that the filamentous structures of the a3 isoform staining were co-localized with microtubules but not with actin filaments. Treatment with cytochalasin D, an inhibitor of actin polymerization, showed no effect on the localization of the a3 subunit. However, no filamentous staining of the a3 isoform was observed by treatment with nocodazole, which causes microtubule depolymerization.5 These results suggest that the a3 iso-

**Fig. 4. Expression of subunit a isoforms during osteoclast differentiation.** A, bone marrow cells were cultured for 7 days in the presence (+) or absence (−) of 1,25(OH)2 D3. Total cell proteins (7 μg) were separated by gel electrophoresis and then incubated with antibodies against a1, a2, or a3, or ones against subunit A (A). Expression of subunit B was essentially the same as that of subunit A (see Footnote 2). B, bone marrow cells were cultured for the indicated numbers of days in the presence of 1,25(OH)2 D3. Cells were subjected to tartrate-resistant acid phosphatase staining to determine the numbers of osteoclasts (OC). Cell nuclei were visualized by staining with methyl green. Scale bar indicates 200 μm. Total proteins (3 μg) were electrophoresed and blotted onto a filter. The immunoblot filter was analyzed by densitometry, and the amounts of the a3 isoform were estimated.

2 T. Toyomura, T. Oka, and M. Futai, unpublished results.
form is associated with microtubules. Subunit A is co-localized with microtubules in avian osteoclast-like cells (44). It is difficult to assume that subunit a alone is transported from the endoplasmic reticulum without assembly as the V_o sector, because subunit a, if not assembled into V-ATPase, is susceptible to proteolytic degradation (1). Thus, V-ATPase (having the a_3 isoform) in the transport vesicles may interact with microtubules and thereby be carried to the ruffled border membrane.

In this regard, microtubules and microfilaments are known to be important for the trafficking of transport vesicles to their destinations (45).

**Acknowledgment**—We thank Dr. K. Kawashima (Teikyo University) for helping us to establish the in vitro culture of osteoclasts.

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J. Biol. Chem. 2000, 275:8760-8765.
doi: 10.1074/jbc.275.12.8760

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