Expression and Localization of the Mouse Homologue of the Yeast V-ATPase 21-kDa Subunit c’’ (Vma16p)*

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Tsuyoshi Nishi, Shoko Kawasaki-Nishi, and Michael Forgac‡
From the Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111

We have identified a cDNA encoding the mouse homologue of the yeast V-ATPase 21-kDa subunit c’’ (Vma16p). The encoded protein contains 205 amino acid residues with five putative membrane spanning segments and shows 48% identity and 64% similarity to the yeast protein. Despite this homology, however, the mouse cDNA does not complement the phenotype of a yeast strain in which the VMA16 gene has been disrupted. Northern blot analysis demonstrated that the 21-kDa subunit is expressed in most tissues examined and showed an expression pattern almost identical to that of the 16-kDa proteolipid subunit (subunit c). The presence of multiple mRNA species suggests the existence of alternatively spliced forms of the 21-kDa subunit which, from Southern blot analysis, are derived from a single gene. Promoter analysis using the luciferase reporter gene revealed that a region 186 bases upstream of the initiation site is sufficient to show a low level of transcriptional activity but that transcription is significantly enhanced by inclusion of the region −186 to −706. The 21-kDa protein was Myc-tagged and the 16-kDa protein was HA-tagged and the tagged proteins were co-expressed in COS-1 cells in order to study their intracellular localization by immunofluorescence microscopy. Both proteins showed significant punctate and perinuclear staining and were predominantly co-localized throughout the cell, consistent with their presence in the same V₀ complexes. Selective permeabilization of cells with digitonin (to permeabilize the plasma membrane) or Triton X-100 (to permeabilize both intracellular and plasma membranes) followed by immunofluorescence microscopy revealed that the carboxyl terminus of the 21-kDa subunit is exposed on the cytoplasmic side of the membrane whereas the carboxyl terminus of the 16-kDa subunit is located on the luminal side of the membrane.

The vacuolar (H⁺)-ATPase (or V-ATPase)1 functions as an ATP-dependent proton pump to acidify intracellular compartments in eukaryotic cells. The V-ATPases are present in a variety of intracellular compartments, including clathrin-coated vesicles, endosomes, lysosomes, Golgi-derived vesicles, chromaffin granules, synaptic vesicles, and the central vacuoles of yeast, Neurospora, and plants (1–8). Vacular acidification plays an important role in many cellular processes, including receptor-mediated endocytosis, intracellular targeting, protein processing and degradation, and coupled transport. In certain mammalian cells, V-ATPases also function in the plasma membrane to transport protons from the cytoplasm to the extracellular environment (9–13). In osteoclasts, plasma membrane V-ATPases play a role in bone resorption (11) whereas in intercalated cells in the kidney they function in renal acidification (9). V-ATPases in the plasma membrane of tumor cells have also been implicated in metastasis (13).

The V-ATPases from fungi, plants, and animals are structurally very similar and are composed of two functional domains termed V₁ and V₀ (1–8). The V₁ domain is a peripheral complex of molecular mass of 570 kDa composed of eight different subunits of molecular masses 79–14 kDa (subunits A–H) that is responsible for ATP hydrolysis. The V₀ domain is a 260-kDa integral complex composed of five subunits of molecular mass 100–17 kDa (subunits a, d, c, c’, and c’’) that is responsible for proton translocation. The V-ATPases are thus similar in structure to the ATP synthases (F-ATPases) of mitochondria, chloroplasts and bacteria (14–19), both in overall structure (20–23) and as revealed by sequence homology of several of the subunits (24–26).

Of the five V₀ subunits, three (subunits c, c’, and c’’) are highly hydrophobic proteins termed proteolipids because of their ability to be extracted with organic solvents (27). Subunits c and c’ are both 16 kDa and are encoded by the VMA3 and VMA11 genes in yeast (28, 29) whereas subunit c’’ is 21 kDa and is encoded by the VMA16 gene (30, 31). All three proteins are homologous to each other and to subunit c of the F-ATPase, although they contain different numbers of putative transmembrane segments. Subunits c and c’ of the V-ATPase contain four putative transmembrane helices and appear to have arisen by gene duplication and fusion of the gene encoding the F-ATPase subunit c (26), which is composed of two transmembrane helices (15). Subunit c’’ (Vma16p) contains an additional putative transmembrane helix at the amino terminus (31). Each of the proteolipid subunits contains an essential buried carboxyl group that is critical for proton transport (31). For the F-ATPase c subunit, this critical carboxyl group is in TM2 (15) whereas for subunits c and c’ of the V-ATPase, the critical residue is located in TM4. Subunit c’’ contains an essential glutamic acid residue in TM3 (31). Mutation of any of these sites completely abolishes proton transport by the V-ATPase, indicating that each V-ATPase complex must contain at least one copy of each of the proteolipid subunits (31).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF356006 and AF356007 (cDNA and genomic DNA encoding the mouse 21-kDa subunit) and AF356008 (cDNA encoding the mouse 16-kDa subunit).
‡ To whom correspondence should be addressed. Tel.: 617-636-6939; Fax: 617-636-0445; E-mail: michael.forgac@tufts.edu.
1 The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; F-ATPase, F,F′-ATP synthase; EST, expression sequence tag; RT-PCR, reverse transcriptase-polymerase chain reaction; RACE, rapid amplification of cDNA ends; HA, hemagglutinin.

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In mammalian cells, a homologue of Vma3p has been identified in mouse (32), bovine (26), and human (33), and recently a homologue of Vma16p (the 21-kDa c′ subunit) has been identified in human (34), although little information concerning this protein has been reported. In this paper, we report the sequence of the mouse Vma16p homologue and demonstrate that its expression pattern and intracellular localization are similar to that of the 16-kDa proteolipid subunit, consistent with its function as a subunit of the V$_0$ domain. Unlike subunit c, however, subunit c′ appears to have a different orientation in the membrane. The possible functional significance of this difference is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials—**Escherichia coli culture media was purchased from Difco Laboratories. Minimal essential medium, fetal bovine serum, and other reagents for tissue culture were purchased from Life Technologies, Inc. Restriction endonucleases, T4 DNA ligase, and other molecular biology reagents were from Life Technologies, Inc., Promega, and New England Biolabs. Most other chemicals were purchased from Sigma.

**Isolation of cDNA Clones—**The cDNA encoding the mouse Vma16p homologue (21 kDa subunit) was identified in the expression sequence tag (EST) clone set of EST clone AA018009 was obtained from the American Tissue Culture Collection (Manassas, VA). The cDNA encoding the mouse Vma3p homologue (16-kDa subunit) (EST clone number AA791459) was also obtained from the American Tissue Culture Collection (Manassas, VA).

**Complementation Test for Mouse Vma16p of the Yeast VMA16 Deletion Strain—**Yeast cells lacking the functional endogenous VMA16 gene were made from YPH500 (MATa ura3-52 lys2-801mmp, ade2-101mms trp1-Δ63 his3-Δ200 leu2-Δ1) by replacing the coding region of the VMA16 gene with the TRP1 gene. The yeast VMA16 gene was integrated into the XbaI and EcoRI site of the pRS413 vector for expression. The mouse 21-kDa subunit gene was inserted just downstream of the VP11 gene promoter and integrated into the 2-µm vector, Yep352. Cells were transformed with the plasmids pRS413, pRS413-VMA16, and Yep352-21K using the lithium acetate method and selected on SD histidine or uracil minus plates. Growth phenotypes of the transfor-
was observed to be essential for V-ATPase activity in yeast (31) and is conserved in all four species.

**The Mouse 21-kDa Subunit Does Not Complement the Function of the Yeast VMA16 Deletion Strain**—To analyze the function of the mouse 21-kDa subunit, we expressed the mouse 21-kDa subunit in yeast cells lacking the endogenous \textit{VMA16} gene. Despite the relatively high identity and similarity between the mouse 21-kDa subunit and the yeast Vma16p (48 and 64%, respectively), the mouse 21-kDa subunit did not complement the growth defect at pH 7.5 of the \textit{VMA16} deletion strain (Fig. 2).

**Tissue Distribution of the Mouse 21-kDa Subunit**—In addition to Vma16p, V-ATPase activity in yeast requires two additional proteolipid subunits (Vma3p and Vma11p), both of which have a molecular mass of \(110\) kDa and contain four putative transmembrane helices (28, 29). A cDNA encoding the mouse homologue of the 16-kDa Vma3p has previously been reported (32). To compare the expression pattern of these two proteolipid subunits in mouse, Northern blot analysis was performed on mRNA isolated from various mouse tissues using RNA probes specific for each subunit. As shown in Fig. 3a, transcripts encoding the 21-kDa subunit were detected in all tissues, with the highest expression detected in heart, brain, liver, kidney, and testis. The signals observed for both proteolipids in skeletal muscle was very low, but this is consistent with the intensity of the band for \(-\text{actin}\). The pattern of expression of the 21-kDa subunit generally paralleled that observed for the 16-kDa subunit. Interestingly, two different size bands were detected by Northern blot in all tissues tested using the probe specific for the 21-kDa subunit (Fig. 3a). The size of the lower band matched that predicted for clone AA018009. This result suggests the existence of alternatively spliced forms of this message. The expression of the 21- and 16-kDa subunits at various developmental stages was also investigated (Fig. 3b), with mRNA for both subunits detectable at all stages tested.

**The Mouse 21-kDa Subunit Is Encoded by a Single Gene**—It was previously reported that there exist several pseudogenes for the 16-kDa subunit in both human (36) and mouse (37). To test whether this was also the case for the 21-kDa subunit and to determine whether the two bands observed for the 21-kDa subunit by Northern blot correspond to alternatively spliced variants of a single gene, Southern blot analysis was performed on mouse genomic DNA digested using several different restriction enzymes. As can be seen in Fig. 4, a single band was observed for each of the restriction digests with the exception of \textit{Pst}\(I\) (see below). This result suggests that the 21-kDa subunit is encoded by a single gene in mouse, without the presence of pseudogenes.

**Genomic Organization of 21- and 16-kDa Subunit Genes**—The gene encoding the mouse 21-kDa subunit was isolated from mouse genomic DNA by PCR genomic walking and was sequenced. As shown in Fig. 5a, the 21-kDa subunit gene...
Tissue distribution of the mouse 21- and 16-kDa subunits. a, membranes to which were transferred 2 µg of poly(A) RNA isolated from mouse heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and testis (lane 8) were hybridized with [32P]dUTP-labeled probes specific for the 21-kDa subunit mRNA, the 16-kDa subunit mRNA, or β-actin mRNA followed by washing and visualization as described under "Experimental Procedures." b, RT-PCR was performed using subunit specific primers with either no template (−) as a negative control or first strand cDNA constructed from RNA derived from embryos of ages 7 to 18 days.

Mouse genomic DNA (10 ng) was digested with EcoRI (lane 1), BamHI (lane 2), PstI (lane 3), and HindIII (lane 4) and separated on a 1% agarose gel. DNA was transferred to an Immobilon-Ny membrane and hybridized with a [32P]dUTP-labeled probe specific for the 21-kDa subunit gene. The position of size markers is indicated to the left of the panel.

Identification of the Transcription Initiation Sites of the Mouse 21- and 16-kDa Subunit Genes—To identify the transcription initiation sites for the 21- and 16-kDa subunit genes, the 5′-RACE was performed using primers specific to each gene and mouse heart cDNA as the template. The 5′-end of the amplified fragments of the 16- and 21-kDa subunit cDNAs are indicated in Fig. 7 as open and closed circles, respectively. As shown, the transcription initiation sites for the 21- and 16-kDa subunit genes are located near the GC boxes at −186 and −218 bp, respectively. Interestingly the basal transcriptional activity of the constructs containing the first 187 bp of 5′-upstream sequence was the same for both genes (13%), suggesting that common elements contribute to this basal activity. Nevertheless, additional sequences between −186 and −706 bp greatly enhance promoter activity. Comparison of the 5′-upstream sequences for these two genes in this region (Fig. 7) reveals the lack of significant sequence homology and the absence of a typical TATA box. However, several putative transcription initiation factor-binding sites, including GC boxes and a CCAAT box, were identified.

Intracellular Localization of the Mouse 21- and 16-kDa Subunits—Although the 21- and 16-kDa subunits show a similar sequence was the same for both genes (13%), suggesting that common elements contribute to this basal activity. Nevertheless, additional sequences between −186 and −706 bp greatly enhance promoter activity. Comparison of the 5′-upstream sequences for these two genes in this region (Fig. 7) reveals the lack of significant sequence homology and the absence of a typical TATA box. However, several putative transcription initiation factor-binding sites, including GC boxes and a CCAAT box, were identified.

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FIG. 6. Promoter activity of the 5'-upstream regions of the 21- and 16-kDa subunit genes. Various lengths of the 5'-upstream regions of the 21- and 16-kDa subunit genes were ligated in front of the luciferase gene in the pGL3-basic plasmid. The pGL3-basic vector has the luciferase gene but no promoter or enhancer sequences and was used as a negative control. The pGL3-control vector contained SV40 promoter and enhancer sequences upstream of the luciferase gene and was used as a positive control. Each of the resulting plasmids was introduced into NIH3T3 cells together with a constant amount of pRL-TK plasmid and the cells incubated for 24 h. Promoter activity was measured and normalized using a dual luciferase assay system. Activity obtained from cells transfected with the pGL3-control vector was defined as 100%. Values represent an average of three independent determinations, with the error bars shown corresponding to the standard deviation. The numbers in each vector name correspond to the number of bases upstream of the initiation codon present.

FIG. 7. Promoter regions of the 21- and 16-kDa subunit genes. Shown are the 5'-upstream regions of the 21- and 16-kDa subunit genes, with identical bases indicated by shading, GC boxes indicated by wavy lines, and a CCAAT box shown with a straight line. The transcription initiation sites for each gene as determined by 5'-RACE are indicated by the closed circles (21-kDa subunit gene) and open circles (16-kDa subunit gene). The previously reported start sites for mRNA from mouse placenta (21-kDa subunit gene) and mouse brain (16-kDa subunit gene) are shown with asterisks.

pattern of expression in different tissues, it is not known whether these proteins form part of the same complex in mammalian cells, as has been demonstrated in yeast (31, 38). To begin to address this question, Myc and HA tags were introduced at the COOH terminus of the 21- and 16-kDa subunits, respectively, and the tagged proteins were expressed in COS-1 cells using the pIRES (CLONTECH) vector. At 36–48 h post-transfection, cells were fixed, permeabilized with 0.5% Triton X-100, and stained with rabbit anti-HA and mouse anti-Myc antibodies. As shown in Fig. 8, both the anti-Myc and anti-HA antibodies showed significant perinuclear staining, with some additional punctate and diffuse staining throughout the remainder of the cell. The merged image (Fig. 8, lower panel) showed extensive overlap in staining with the anti-Myc and anti-HA antibodies, indicating co-localization of the 21- and 16-kDa subunits.

To determine whether the expressed proteolipids co-localized with other V-ATPase subunits, COS-1 cells were transfected with the HA-tagged form of the 16-kDa subunit (Fig. 9, panels a and b) and the Myc-tagged (Fig. 9b) or HA-tagged (Fig. 9c) forms of the 21-kDa subunit. Cells were then stained with an antibody against the HA-tag to identify the proteolipid subunits, and with the monoclonal antibody 3.2-F1 against the A subunit (part of the V1 domain). As can be seen in Fig. 9, distinct patterns of perinuclear and punctate staining were observed for the proteolipid subunits as compared with the A subunit, with little overlap between the patterns. This result suggests that the expressed proteolipid subunits, while assembling together to form V0 domains, may not be able to assemble with the V1 domain, or that the antibodies against the epitope tags are only able to recognize their binding sites in the free V0 domain but not in fully assembled V-ATPase complexes (see “Discussion”).

Localization of the Carboxy-terminal of the 21- and 16-kDa Subunits—From the deduced amino acid sequence, the 21-kDa subunit is predicted to have five transmembrane segments whereas the 16-kDa subunit is predicted to contain four membrane spans. To address the membrane topology of these subunits, we analyzed the orientation of the carboxyl-terminal of these proteins. HA-tagged forms of either the 21- or 16-kDa subunits were expressed in COS-1 cells (the HA epitope tag in both cases was attached at the carboxyl terminus of the protein). Cells were then differentially permeabilized with either Triton X-100 (which permeabilizes both intracellular and plasma membranes) or low levels of digitonin (under which conditions the plasma membrane becomes permeabilized but intracellular membranes remain intact (39)), followed by immuno-fluorescent staining using antibodies against either HA or an antibody against subunit A of the V1 domain. As shown in Fig. 10, permeabilization of cells with Triton X-100 led to staining of both the HA-tagged 21-kDa subunit (panel f) and the 16-kDa subunit (panel b) as well as subunit A (panels a and c). By contrast, permeabilization of cells with digitonin led to staining of the HA-tagged 21-kDa subunit (panel h) and subunit A (panels c and g), but not the 16-kDa subunit (panel d). These results suggest that the carboxy-terminal 21-kDa subunit is facing the cytoplasmic side of the membrane whereas the carboxy-terminal of the 16-kDa subunit is oriented toward the lumenal side of the membrane.

DISCUSSION

The yeast V-ATPase contains three distinct proteolipid subunits: subunit c (Vma3p), subunit c¢ (Vma11p), and subunit c¢’ (Vma16p). Mutagenesis studies have revealed that each of
the 21-kDa homologue of Vma3p has been reported for several species, including C. elegans, bovine, and human (26, 32, 33), and disruption of the corresponding gene has been shown to lead to embryonic lethality in mouse (37, 63). A BLAST search against the human genomic sequence database indicates that the 16-kDa subunit Vma3p homologue found in C. elegans (35) and Drosophila melanogaster (38), but not in vertebrates. These results suggest that the mammalian V-ATPase may have only two proteolipid subunits, namely the homologues of Vma3p and Vma16p. It should be noted that subunit c has also been shown to associate with several other cellular targets, including the E5 oncoprotein (47) and β1-integrin (48), and overexpression of this subunit has been shown to lead to cell transformation (49). It is thus possible that the proteolipid subunits may play roles in mammalian cells in addition to their role in V-ATPase activity.

By contrast with the Vma3p homologue, relatively little is known about the 21-kDa Vma16p homologue in animal cells. The sequence of both the human and the mouse Vma11p homologues has been shown to lead to embryonic lethality in mouse (37, 63). A BLAST search against the human genomic sequence database indicates that the 16-kDa subunit Vma3p homologue is located on chromosome 6, while three pseudogenes (previously reported as VP-2, VP-4, and VP-6 (36)), are located on chromosomes 1, 17, and 3, respectively. Interestingly, although a homologue to Vma16p has been identified in the human genome on chromosome 1, no homologue of Vma11p has been found. Thus, a Vma11p homologue has been identified in C. elegans (35) and Drosophila melanogaster (38), but not in vertebrates.
In the current report we have compared the gene structure and expression patterns of the mammalian 21- and 16-kDa subunits and have provided the first information concerning their intracellular localization and orientation in the membrane. The deduced amino acid sequence of mouse 21-kDa subunit is 95% identical to that of human ATP6F (34), 58% identical to VHA4 of C. elegans (35), and 48% identical to Vma16p from yeast (30), with the highest degree of homology residing in the four COOH-terminal most transmembrane helices (Fig. 1). This high degree of sequence conservation is consistent with a critical role of the 21-kDa subunit in V-ATPase function. Nevertheless, the mouse cDNA is unable to complement the growth phenotype of yeast cells disrupted in ATPase function. The mouse cDNA expresses the human 16-kDa subunit, whereas the mouse cDNA expresses the human 16-kDa subunit (Fig. 5) reveals that, despite considerable sequence homology, certain exons on the 16-kDa subunit gene have been divided in the 21-kDa subunit gene (Fig. 11). Thus exon II of the 16-kDa subunit gene (which encodes TM2 and the TM1/2 and TM2/3 loops and is shown in green) has been divided into exons IV, V, and VI in the 21-kDa subunit gene, with exon V encoding most of TM3 and the bordering exons encoding the polar loops. Similarly, exon III of the 16-kDa subunit gene (which encodes both TM3 and TM4 and is shown in blue) has been divided into exons VII and VIII in the 21-kDa subunit gene. By contrast, exon I of the 16-kDa subunit gene (encoding TM1 and shown in yellow) appears to be directly related to exon III of the 21-kDa gene (encoding TM2). Thus, the extra sequence present in the 21-kDa protein (shown in red) appears to correspond to the amino-terminal most transmembrane helix (encoded by exons I and II). The function of the amino-terminal transmembrane segment is an important but unanswered question, but one possibility is that it may occupy the central cavity in the ring of c subunits in V_o.

Although the similar pattern of expression of the 21- and 16-kDa subunits suggests common elements controlling transcription, comparison of the 5’-upstream regions does not reveal any significant homology beyond the presence of several GC boxes (Fig. 7). The results of reporter gene assays (Fig. 6) and identification of the transcription initiation sites (Fig. 7) suggests that the proximal GC box immediately upstream of the initiation codon may be sufficient for the start of transcription from both genes, but that elements in the region −180 to −700 bp greatly enhance transcription. Interestingly the 5’ end
of the 21-kDa subunit mRNA from both heart (reported here) and placenta (from the EST data base) are very similar (Fig. 7), whereas the start sites for the 16-kDa subunit mRNA from heart and brain (32) are separated by nearly 100 bp, suggesting possible tissue specific control of transcription initiation.

The co-localization of the 21- and 16-kDa subunits by immunofluorescence (Fig. 8) is consistent with these proteins being present in the same V$_0$ complexes, although the failure of the expressed proteins to co-localize with the A subunit of the V$_1$ domain (Fig. 9) suggests that the tagged proteins are not assembling into fully functional V-ATPase complexes, possibly because of interference with assembly by the epitope tags. Alternatively, the epitope tags on the proteolipid subunits may only be exposed in the free V$_0$ domains but not in fully assembled V-ATPase complexes. This has been observed for the monoclonal antibody 10-D7 against the 100-kDa a subunit of the yeast V-ATPase, which recognizes its epitope only in the free V$_0$ domain but not in the assembled V-ATPase (54). It is also possible that overexpression of the proteolipids may lead to the production of an excess of V$_0$ domains over what is required to form functional V-ATPase complexes, although the lack of co-localization of the proteolipids and subunit A would require that such newly assembled V$_0$ complexes exchange V$_1$ domains very slowly with pre-existing complexes. Such slow exchange of free V$_1$ and V$_0$ complexes into assembled complexes under steady state conditions has been reported for Madin-Darby bovine kidney cells (55). Finally, it is possible that overproduction of the proteolipids leads to their aberrant localization to the endoplasmic reticulum, where many misfolded proteins are targeted, although the pattern of staining observed in Fig. 9 is not that typically observed for endoplasmic reticulum in COS-1 cells, which show a much more reticular pattern surrounding the entire nucleus (56). The co-localization of the proteolipid subunits is interesting in light of the recent reports of association of the 16-kDa protein with other cellular targets, such as the E5 oncprotein (47) and platelet-derived growth factor receptor by the V-ATPases (31). Hence these polar loops of the 21-kDa subunit would not be available to interact with the V$_1$ domain and the helix containing the prototypical gate involved in proton transport would traverse the membrane with an opposite orientation to the other carboxyl bearing transmembrane segments in the ring of proteolipid subunits. Because the carboxyl group in TM3 does have an adjacent polar loop exposed on the cytoplasmic side of the membrane (but immediately COOH-terminal rather than NH$_2$-terminal), whereas the carboxyl group in TM5 does not, this may explain why the carboxyl group in TM5 of Vma16p is the critical residue for proton transport. Further work will be required to test our model for the folding of the 21-kDa subunit in the membrane.

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Tsuyoshi Nishi, Shoko Kawasaki-Nishi and Michael Forgac

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