Expression and Function of the Mouse V-ATPase d Subunit Isoforms*

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We have identified a cDNA encoding a novel isoform of the mouse V-ATPase d subunit (d2). The protein encoded is 350 amino acids in length and shows 42 and 67% identity to the yeast d subunit (Vma6p) and the mouse d1 isoform, respectively. Reverse transcriptase-PCR analysis using isoform-specific primers demonstrate that d2 is expressed mainly in kidney and at lower levels in heart, spleen, skeletal muscle, and testis. Although d1 and d2 show similar levels of sequence homology to Vma6p, only the d1 isoform can complement the phenotype of a yeast strain in which VMA6 has been disrupted when cells are grown at 30 °C. The d2 isoform, however, can complement the vma6Δ phenotype when cells are grown at 25 °C. Moreover, partial assembly of the V-ATPase complex on the vacuolar membrane can be detected under these conditions, although assembly is significantly lower than that observed for the strain expressing Vma6p. This reduced assembly is also reflected in a reduced level of concanamycin-sensitive ATPase activity and proton transport in isolated vacuoles. Comparison of the kinetic properties of V-ATPase complexes containing Vma6p and d1 demonstrate that although the $K_m$ for ATP hydrolysis is similar (0.26 and 0.31 mM, respectively), the coupling ratio (proton transport/ATP hydrolysis) is ∼3–6-fold higher for d1-containing complexes than for Vma6p-containing complexes. These results suggest that subunit d may play a role in coupling of proton transport and ATP hydrolysis.

The vascular (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). Vascular 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, V1 and V0 (1–8). The V1 domain is a peripheral complex with molecular mass of 640 kDa composed of eight different subunits of molecular mass 70–14 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 masses 100–17 kDa (subunits a, d, c, c', and c") that is responsible for proton translocation. In yeast cells, all subunits are encoded by single genes with the exception of subunit a, which is encoded by two genes, VPH1 and STV1 (14, 15).

Information about the function of four of the five V0 subunits has been obtained by site-directed and random mutagenesis. Thus, each of the three proteolipid subunits (c, c', and c") have been shown to contain a single, buried glutamate residue that is essential for proton translocation (16, 17). In addition, the a subunit contains a number of buried charged residues that influence proton transport (18–20), as well as a single buried arginine residue in transmembrane segment seven, which is required for proton translocation (20). Subunit a has also been shown to play a role in intracellular targeting of the V-ATPase (15, 21) and in controlling assembly with V1, coupling of proton transport and ATP hydrolysis, and reversible dissociation of the V1 and V0 domains in response to glucose depletion (21, 22). Despite this information regarding most of the V0 subunits, virtually nothing is known about the function of subunit d. The gene encoding subunit d was initially cloned from bovine adrenal medulla (23) and subsequently from yeast as the VMA6 gene, where its disruption was shown to lead to a typical Vma-phenotype (24). Sequence analysis revealed the presence of no obvious transmembrane segments (23, 24), despite the fact that it remains firmly attached to the V0 domain upon dissociation of V1 using chaotropic agents (25) or, in vivo, upon glucose withdrawal (26). This suggests that subunit d is bound to the V0 domain through protein-protein interactions rather than by integration into the membrane. Mild proteolysis results in rapid cleavage of subunit d in intact clathrin-coated vesicles, suggesting that it is exposed on the cytoplasmic side of the membrane (27).

In addition to yeast and bovine, the gene encoding subunit d has also been cloned and sequenced from humans and plants (28, 29). Like numerous other V-ATPase subunits in higher eukaryotes, subunit d appears to be encoded by multiple genes (28). In this paper, we have identified a novel isoform of subunit d in mouse, investigated its pattern of expression, and...
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EXPERIMENTAL PROCEDURES

Materials—Escherichia coli culture media was purchased from Difco Laboratories, restriction endonucleases, T4 DNA ligase, and other molecular biology reagents were from Invitrogen, Promega, and New England Biolabs. Most other chemicals were purchased from Sigma.

Isolation of DNA Clones—The cDNA encoding the mouse d subunit isoform d2 was identified in the expression sequence tag data base and expressed sequence tag clone uj32h10 was obtained from the American Type Culture Collection. The cDNA encoding the mouse d1 isoform (uj10a07) was also obtained from the American Type Culture Collection.

Test of the Ability of the Mouse d Subunit Isoforms to Complement the Phenotype of the Yeast VMA6 Deletion Strain—Yeast cells lacking the functional endogenous VMA6 gene were made from YPH500 (MATα ura3–52 lys2–801 trp1–901 ade2–101 his3–200 leu2–3,112 trp1) by replacing the coding region of VMA6 with the TRP1 gene. The yeast VMA6 gene was isolated from the YPH500 genomic DNA by PCR using the following primers: forward, ctccagcatacatattaatgtgca; reverse, gtccagttgagccgaggatattact. The amplified fragments were cloned into the pRS413 vector. The yeast gene was sequenced.

RESULTS

Identification of the cDNA Encoding the Mouse V-ATPase d2 Subunit Isoform—The cDNA sequence encoding the d2 subunit of the V-ATPase was first reported in bovine (23), and was subsequently identified in other species (24, 33–37). Genomic sequencing has recently demonstrated the existence of a second isoform of the V-ATPase d subunit, d2, which has been identified in humans (28) and plants (29). A search of the mouse expressed sequence tag and genome data bases revealed several clones encoding the second isoform of subunit d in mouse. One of these clones (uj32h10) was completely sequenced and the deduced amino acid sequence was aligned with the other d subunit isoforms, d1, and the yeast d subunit, Vma6p (Fig. 1). The protein encoded contains 350 amino acid residues and has a predicted molecular mass of 40,459. The gene encoding the mouse d1 isoform is located on chromosome 4, whereas the gene encoding d2 is located on chromosome 8. Based on the genomic sequence, the genes encoding the d1 and d2 isoforms, RT-PCR was performed using isoform-specific primers and RNA isolated from eight mouse tissues and several developmental stages of mouse embryos.

Vacuole Membrane Isolation—Subcellular fractionation of organelles from yeast cells expressing Vma6p, the mouse d1 isoform, or the mouse d2 isoform was performed by differential centrifugation of cell lysates as described previously (30, 31). Yeast cells were cultured in SD-HIS medium to an A600 of 1 at 30 or 25°C. Cells (3 × 107) were pelleted, resuspended in 0.7 M sorbitol, 2 mM dithiothreitol, 100 mM Mes-Tris (pH 7.5), and 50 µg/ml Zymolase 100T, and incubated at 30°C with gentle shaking for 15 min. Cells were pelleted, resuspended in YEFD medium containing 0.7 M sorbitol, 2 mM dithiothreitol, 100 mM Mes-Tris (pH 7.5), and 50 µg/ml Zymolase 100T, and incubated at 30°C with gentle shaking for 60 min. Spheroplasts were washed with YEPD medium containing 0.7 M sorbitol, 2 mM dithiothreitol, osmotically lysed by incubation in lysis buffer (50 mM Tris-HCl (pH 7.5) 0.2 M sorbitol, 1 mM EDTA), and the vacuoles were isolated by flotation on two consecutive Ficoll gradients as previously described (22). Protein concentrations were measured by the BCA protein assay (Pierce).

Detection of V-ATPase Subunits Present on Isolated Vacuolar Membranes—Vacuolar membranes were isolated from the yeast vma6Δ strain transformed with pRS413-VMA6, pRS413-TEF-d1, or pRS413-TEF-d2 and were subjected to SDS-PAGE followed by Western blot analysis using the mouse monoclonal antibodies 10D7 against Vph1p, 8B1-F3 against Vma1p, and 13D11-B2 against Vma2p (Molecular Probes, Inc.). Blots were also probe with rabbit polyclonal antibodies against Vma6p, Vma7p, Vma8p, Vma10p, and Vma13p (all generously provided by Dr. Tom Stevens), Vma4p (a generous gift of Dr. Dan Klionsky), and Vma6p (a generous gift of Dr. Patricia Kane). Following removal of unbound primary antibodies by washing, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and developed using a chemiluminescence detection method obtained from Kirkegaard and Perry Laboratories.

Other Methods—Protein concentrations were determined by the BCA protein assay (Pierce). ATPase activity was measured using a coupled spectrophotometric assay in the presence or absence of 1 µM concanamycin, as previously described (22). ATP-dependent proton transport was measured in transport buffer (25 mM Mes-Tris (pH 7.2) 5 mM MgCl2) using the fluorescence probe 9-amino-6-chloro-2-methoxyxuridine in the presence or absence of 1 µM concanamycin, as previously described (22). SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (32).
the d2 isoform is expressed predominantly in kidney, although some expression is also detected in lung, testis, skeletal muscle, and heart, in agreement with previous observations (23). With respect to developmental stage, the d1 isoform is expressed throughout early development, whereas the d2 isoform is expressed heavily at day 7 followed by a disappearance and a gradual recovery to original levels by day 17. Development of the mouse kidney begins after embryonic day 14. These results suggest that expression of the d2 isoform from day 15 is correlated with kidney development. Expression of the d2 isoform at day 7 suggests that this isoform may also serve some other function in early mouse development.

The 5′/H11032-Untranslated Region of the Mouse and Human d Subunit Isoforms—To address the basis for the tissue-specific expression of the d2 isoform, the nucleotide sequence of 5′/H11032-upstream regions of the mouse and human genes were compared. The 5′/H11032-upstream sequence of each gene was identified from human and mouse genome sequence data bases reported at NCBI. Harr-plot analysis indicates that although the 5′/H11032-upstream regions of the genes encoding the mouse d1 and d2 isoforms have no obvious homology (data not shown), both of the d1 and d2 5′/H11032-upstream regions show significant sequence homology between mouse and human (Fig. 4). The transcription initiation sites were identified using 5′/H11032-rapid amplification of cDNA ends, primers specific for each gene and mouse kidney cDNA as the template and are shown in Fig. 4, a and b, by closed circles. As can be seen, for the gene encoding the d1 these sites fall within one of the two regions of high sequence conservation, whereas for the gene encoding d2, transcription starts just downstream of the 3′ most region of homology.

The 5′-Untranslated Region of the Mouse and Human d Subunit Isoforms—To address the basis for the tissue-specific expression of the d2 isoform, the nucleotide sequence of 5′-upstream regions of the mouse and human genes were compared. The 5′-upstream sequence of each gene was identified from human and mouse genome sequence data bases reported at NCBI. Harr-plot analysis indicates that although the 5′-upstream regions of the genes encoding the mouse d1 and d2 isoforms have no obvious homology (data not shown), both of the d1 and d2 5′-upstream regions show significant sequence homology between mouse and human (Fig. 4). The transcription initiation sites were identified using 5′-rapid amplification of cDNA ends, primers specific for each gene and mouse kidney cDNA as the template and are shown in Fig. 4, a and b, by closed circles. As can be seen, for the gene encoding the d1 these sites fall within one of the two regions of high sequence conservation, whereas for the gene encoding d2, transcription starts just downstream of the 3′ most region of homology.

Complementation of the Yeast vma6Δ Phenotype by the Mouse d Subunit Isoforms at 30 °C—To analyze the function of the mouse d subunit isoforms, we expressed each of these isoforms in a yeast strain (vma6Δ) in which the endogenous VMA6 gene has been disrupted. Despite the very similar level of sequence identity between the two mouse proteins and
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FIG. 4. 5’-Upstream regions and transcription initiation sites of the genes encoding the mouse d1 and d2 subunit isofoms. Nucleotide sequence of the 5’-upstream regions of the genes encoding the mouse d1 (a) and d2 (b) subunits (accession numbers NW_000349 and NW_000354). Regions highly homologous between mouse and human (d1, NT_010478 and d2, NT_008117) sequences are indicated by boxes. The closed circles represent transcription initiation sites, which were identified by 5’-rapid amplification of cDNA ends analysis using mouse kidney RNA, are indicated by closed circles. The number of circles indicate the number of 5’-ends obtained by this analysis.

Vma6p (45 and 42% for d1 and d2, respectively), only d1 was able to complement the growth defect of this strain when assayed at pH 7.5 and 30 °C (Fig. 5a). There are several possible reasons to account for the inability of the d2 isoform to complement the Vma- phenotype of the vma6Δ strain, including loss of activity of the V-ATPase and failure to assemble a complex. As an initial assessment of the assembly competence of the heterologously expressed proteins, Vma6p, the mouse d1 isoform or the mouse d2 isoform were expressed in the yeast Vma- strain expressing either Vma6p, the mouse d1 isoform or the mouse d2 isoform after growth at 25 °C. Concanamycin-sensitive ATPase activity was then measured over a range of ATP concentrations from 0.05 to 2.0 mM using a coupled spectrophotometric assay and concanamycin-sensitive, ATP-dependent proton transport was measured from the initial rate of fluorescence quenching using the fluorescence dye 9-amino-6-chloro-2-methoxyacridine. The tightness of coupling of proton transport obtained with the d1 isoform, however, was 22% of that obtained with Vma6p, when measured at the same protein concentration. This gave a coupling ratio for the d1-
**TABLE I**

| d subunit isofoms | ATPase activity | Initial rate of fluorescent quenching | Coupling ratio (fluorescent quenching/ATPase activity) |
|-------------------|-----------------|--------------------------------------|------------------------------------------------------|
|                   | μmol ATP/μmol ATPmin | 0.5 mM | 5 μM | 0.5 mM |
| Wild              | 300 ± 0.006       | 0.008 | 9.5 ± 0.11 | 13.4 (24.6) |
| d1                | 306 ± 0.006       | 0.006 | 2.09 ± 0.86 | 85.4 |
| d2                | ND               | ND    | 0.52 ± 0.19 | ND |

*Measured as described in Ref. 22.

*Coupling ratio of wild type vacuolar membranes diluted 21-fold to give a level of ATPase activity similar to that measured for d1-containing vacuolar membranes.

*ND, not detected.

containing vacuoles of 80.4, −6-fold higher than for the wild type vacuoles (13.4). If the wild type vacuoles were diluted 21-fold to give approximately the same level of ATPase activity as was observed for the d1 mutant, a coupling ratio of 24.6 was obtained, corresponding to a 3-fold difference in coupling ratio between the wild type and d1 mutant. These results suggest that the V-ATPase complex containing the mouse d1 isoform was more tightly coupled than the wild type complex. Whereas ATPase activity obtained with the d2 isoform was below the detection level, some concanamycin-sensitive proton transport was still observed.

**Assembly of the V-ATPase Complexes Containing the Mouse d1 or d2 Isoforms**—To further evaluate the assembly status of V-ATPase complexes containing the mouse d1 and d2 isoforms, vacuoles were isolated from cells grown at 25 °C, and Western blotting was performed using antibodies against subunits A, B, C, D, E, F, G, and H in the V_0 domain and subunit a in the V_1 domain. As can be seen in Fig. 7, the d1 isoform showed reduced levels of V-ATPase subunits on vacuolar membranes relative to Vma6p. For the d2 isoform, there was an even greater reduction in subunit levels. These results suggest that although the V-ATPase complexes containing the d2 isoform are sufficiently active to complement the Vma- phenotype of the vma6Δ strain and sufficiently stable to show partial assembly using the fractionation procedure employed in Fig. 6, they are not sufficiently stable to survive the conventional vacuole isolation procedure used to prepare vacuolar membranes for the experiment shown in Fig. 7.
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Of the five V₀ subunits, the least characterized in terms of function is subunit d. Thus all three proteolipid subunits (c, c', and c) as well as subunit a have been shown to participate in proton translocation (16–20). Subunit d has also been shown to function in intracellular targeting and in control of coupling efficiency and in vivo dissociation (15, 21, 22). Although disruption of the gene encoding subunit d in yeast (Vma6p) prevents assembly of both the intact complex and the V₀ domain (24), no specific function has yet been assigned to this subunit. Subunit d does not appear to be embedded in the lipid bilayer (23, 24), but is instead exposed on the cytoplasmic side of the membrane (27), where it can be released from the membrane by treatment at alkaline pH or with urea (24). It may, therefore, contribute to the cytoplasmic mass observed in electron microscopic images of the V₀ domain (40).

Multiple isoforms have been identified for several of the V-ATPase subunits in higher eukaryotes, including subunits B, C, E, G, and a (11, 28, 39, 41–47). Recently, two isoforms of subunit d have been identified in humans and plants (28, 29). We have isolated a cDNA encoding a second isoform of subunit d in mouse (d2), and have demonstrated that it is expressed predominantly in kidney, although expression is also detected in lung, testis, skeletal muscle, heart, and spleen (Fig. 3). This expression pattern is similar to that observed for two other subunit isoforms, namely B1 and a4, which have been suggested to be "kidney specific" (41, 42, 47, 48). Comparison of the 5'-upstream regions of the mouse and human genes encoding the d1 and d2 isoforms reveals the presence of several isoform-specific regions that are conserved between species (Fig. 4). These regions may play a role in tissue-specific expression of the corresponding genes (49). Consistent with this idea, the sites of transcription initiation are located in or near these conserved regions. Previously, the 5'-upstream region of the genes encoding subunits c, c', and B2 were analyzed and possible promoter sequences or cis-elements were reported (50, 51). However, no homology is observed in the 5'-upstream regions of these genes when compared with those encoding the d subunit isoforms (not shown).

To try to gain insight into the function of subunit d, each mouse d subunit isoform was expressed in a yeast strain disrupted in VMA6. Interestingly, the d1 isoform is able to complement the Vma- phenotype of this strain at both 25 and 30 °C, whereas the d2 isoform complemented the phenotype only at the lower temperature. Western blot analysis of vacuole-enriched membranes detected no assembly of V-ATPase complexes in the strain expressing d2 at 30 °C (Fig. 5), indicating that either d2 is not stable or the V-ATPase complexes containing d2 are not stable under these conditions. By contrast, assembly of d2-containing complexes on vacuole-enriched membranes is detected at 25 °C, although at lower levels than for cells expressing either Vma6p or d1 (Fig. 6). Moreover, d2-containing complexes display greatly reduced stability, even at 25 °C, as indicated by the very low levels of V-ATPase subunits present on purified vacuolar membranes (Fig. 7). Assembly of d1-containing complexes is observed at both 25 and 30 °C, although the level of V-ATPase subunits on purified vacuolar membranes is lower for cells expressing d1 than for Vma6p-expressing cells (Fig. 7). These results indicate that both mouse isoforms are able to replace the yeast d subunit in assembly of V-ATPase complexes, although the d1 isoform is more effective in this capacity. A temperature-dependent difference in the ability of E subunit isoforms from mouse to replace the yeast VMA4 gene has also been reported (45).

Consistent with the above results, V-ATPase complexes containing the d1 isoform showed much lower levels of concanamycin-sensitive ATPase activity than Vma6p-containing complexes, and virtually no V-ATPase activity was detectable on purified vacuolar membranes from d2-expressing cells. It was therefore not possible to characterize the activity properties of V-ATPase complexes containing d2. Interestingly, V-ATPase complexes containing the d1 isoform showed 3–6-fold greater coupling of proton transport to ATP hydrolysis than Vma6p-containing complexes (Table I). These results suggest that the yeast V-ATPase complex is not optimally coupled under normal conditions, in agreement with previous results (52). In that study, a mutation was identified in the "non-homologous" region of the yeast A subunit that resulted in a 4-fold increase in coupling efficiency relative to the wild type enzyme. Mutations altering coupling of proton transport to ATP hydrolysis have also been identified in a number of other V-ATPase subunits, including subunits D and a (53, 20), and a 5-fold difference in coupling ratio has been reported for V-ATPase complexes containing different a subunit isoforms (22). These results suggest that a number of V-ATPase subunits in both the V₁ and V₀ domains act in coordinate fashion to control coupling of proton transport and ATP hydrolysis, and that subunit d may also function in this capacity.

While this manuscript was in preparation, another paper describing the identification of a second isoform of subunit d in mouse appeared (54). Our results are in agreement with this paper in demonstrating high expression of d2 in kidney and in showing that only the d1 isoform was able to complement the Vma- phenotype of the vma6Δ strain at 30 °C. We have extended these results in the current article by demonstrating a temperature-dependent ability of the d2 isoform to complement the Vma- phenotype of the vma6Δ strain and by characterizing the assembly and activity properties of the hybrid yeast complexes containing the mouse d subunit isoforms.

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