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**ATP6AP1** deficiency causes an immunodeficiency with hepatopathy, cognitive impairment and abnormal protein glycosylation

Eric J.R. Jansen¹,*, Sharita Timal²,³,*, Margret Ryan⁴,*, Angel Ashikov²,³, Monique van Scherpenzeel²,³, Laurie A. Graham⁴, Hanna Mandel⁵, Alexander Hoischen⁶, Theodore C. Iancu⁷, Kimiyo Raymond⁸, Gerry Steenbergen³, Christian Gilissen⁶, Karin Huijben³, Nick H.M. van Bakel¹, Yusu Ke Maeda⁹, Richard J. Rodenburg³,¹⁰, Maciej Adamowicz¹¹, Ellen Crushell¹², Hans Koenen¹³, Darius Adams¹⁴, Julia Vodopiutz¹⁵, Susanne Greber-Platzer¹⁵, Thomas Müller¹⁶, Gregor Dueckers¹⁷, Eva Morava¹⁸,¹⁹,²⁰, Jolanta Sykut-Cegielska²¹, Gerard J.M. Martens³, Ron A. Wevers³, Tim Niehues¹³, Martijn A. Huynen²², Joris A. Veltman⁶,²³, Tom H. Stevens⁴ & Dirk J. Lefeber²,³

The V-ATPase is the main regulator of intra-organellar acidification. Assembly of this complex has extensively been studied in yeast, while limited knowledge exists for man. We identified 11 male patients with hemizygous missense mutations in **ATP6AP1**, encoding accessory protein Ac45 of the V-ATPase. Homology detection at the level of sequence profiles indicated Ac45 as the long-sought human homologue of yeast V-ATPase assembly factor Voa1. Processed wild-type Ac45, but not its disease mutants, restored V-ATPase-dependent growth in Voa1 mutant yeast. Patients display an immunodeficiency phenotype associated with hypogammaglobulinemia, hepatopathy and a spectrum of neurocognitive abnormalities. Ac45 in human brain is present as the common, processed 40-kDa form, while liver shows a 62-kDa intact protein, and B-cells a 50-kDa isoform. Our work unmasks Ac45 as the functional ortholog of yeast V-ATPase assembly factor Voa1 and reveals a novel link of tissue-specific V-ATPase assembly with immunoglobulin production and cognitive function.

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¹Department of Molecular Animal Physiology, Donders Institute for Brain, Cognition and Behaviour, Centre for Neuroscience and Radboud Institute for Molecular Life Sciences, Faculty of Science, Radboud University, 6525 GA Nijmegen, The Netherlands. ²Department of Neurology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands. ³Department of Laboratory Medicine, Translational Metabolic Laboratory, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands. ⁴Department of Chemistry and Biochemistry, Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403, USA. ⁵Metabolic Unit, Ramberg Health Care Center, Rappaport School of Medicine, Technion, 3190601 Haifa, Israel. ⁶Department of Human Genetics, Radboud Institute for Molecular Life Sciences and Donders Centre for Neuroscience, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands. ⁷The Milman-David Biomedical Research Unit, 24 Hazevi Avenue, 34355 Haifa, Israel. ⁸Department of Laboratory Medicine and Pathology, Mayo College of Medicine, Rochester, Minnesota 55905, USA. ⁹Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan. ¹⁰Department of Pediatrics, Nijmegen Centre for Mitochondrial Disorders (NCMD), Radboud university medical center, 6525 GA Nijmegen, The Netherlands. ¹¹Protein Laboratory, Children’s Memorial Health Institute, 04730 Warsaw, Poland. ¹²Temple Street Children’s University Hospital, Temple Street, Dublin 1, DC01 YC67, Ireland. ¹³Department of Laboratory Medicine, Medical Immunology, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands. ¹⁴Personalized Genomic Medicine Pediatric Genetics and Metabolism Goryeb Children’s Hospital, Morristown, New Jersey 07960, USA. ¹⁵Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, 1090 Vienna, Austria. ¹⁶Department of Pediatrics I, Medical University of Innsbruck, 6020 Innsbruck, Austria. ¹⁷HELIOS Klinikum Krefeld, Children’s Hospital, Lutherplatz 40, 47805 Krefeld, Germany. ¹⁸Department of Pediatrics, Tulane University Medical School, New Orleans, Los Angeles 70112, USA. ¹⁹Department of Pediatrics, University Medical School of Leuven, 3000 Leuven, Belgium. ²⁰Department of Pediatrics, Radboudumc, 6525GA, Nijmegen, The Netherlands. ²¹Screening Department, Institute of Mother and Child, 01-211 Warsaw, Poland. ²²Centre for Molecular and Biomolecular Informatics, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6525GA Nijmegen, The Netherlands. ²³Department of Clinical Genetics, Maastricht University Medical Centre, 6229HX Maastricht, The Netherlands. * These authors contributed equally to this work. Correspondence and requests for materials should be addressed to T.H.S. (email: tstevens@uoregon.edu) or to D.J.L. (email: DirkLefeber@radboudumc.nl).
The vacuolar H^+-ATPase (V-ATPase) is a ubiquitously expressed protein complex, required for luminal acidification of secretory vesicles to acidify the extracellular milieu, compartments of the endocytic pathway including lysosomes, and of the Golgi apparatus. The V-ATPase consists of two multi-protein domains, V_1 and V_0. The peripheral V_1 domain comprises eight subunits (A–H), is localized in the cytoplasm and hydrolyses ATP. The V_0 domain is embedded in the organelle membrane, consists of five subunits (a, d, e, c and c') and harbours the rotary mechanism for proton translocation. Human disease mutations in V-ATPase core subunits result in distinct clinical syndromes. In 1999, renal tubular acidosis with deafness was the first phenotype linked to the V-ATPase with mutations in the kidney-specific isoforms ATP6V1B1 (MIM 267300) or ATP6V0A4 (MIM 602722) (refs 3–5). In 2000, osteopetrosis (MIM 259700) was linked to the V-ATPase by identification of mutations in TCIRG1, encoding the osteoclast-specific a3 subunit. In 2008, mutations were found in ATP6V0A2 in a subgroup of cutis laxa syndromes with abnormal protein glycosylation. The V-ATPase assembly factor Voa1 mutant yeast with the processed C-terminal domain of Ac45. Identification of different Ac45 protein isoforms in human vertebrates, with Ac45 ubiquitously expressed with the V-ATPase assembly factor Voa1 was predicted to be homologous to the yeast vacuolar ATPase assembly factor (Voa1) and is encoded in the processed C-terminal domain of Ac45. The dominating clinical phenotype of Ac45 deficiency. The clinical phenotype of Ac45 deficiency. The clinical phenotype of Ac45 deficiency. The clinical phenotype of Ac45 deficiency. The clinical phenotype of Ac45 deficiency.

Results

Identification of mutations in X-linked ATP6AP1. In our cohort of unsolved patients with deficient glycosylation of proteins, we performed exome sequencing to identify the causative gene defect. Exome sequencing of a male patient (individual 1.1, Table 1) was performed as previously described. After filtering out poor-quality variants as well as common and synonymous variants (see Methods section), 131 rare missense variants were selected. Based on a recessive inheritance model, two candidate genes remained (Supplementary Table 1): X-linked ATP6AP1 with hemizygous variant and KPRP on Chr1 with compound heterozygous variants. Of these variants, the c.1284G>A variant in ATP6AP1 on chrXq28 showed the highest level of conservation (PhyloP 46-way, 5.1) and was predicted to be pathogenic by Sift, Polyphen-2 and MutationTaster. Moreover, no potentially pathogenic variants were identified in KPRP in the WES data of patients 2.1 and 6.1. ATP6AP1 encodes the accessory subunit Ac45 of the V-ATPase complex, the proton pump that has been linked with abnormal glycan processing in the Golgi via mutations in its core subunit ATP6V0A2 (ref. 8). Sanger sequencing confirmed the hemizygous missense mutation (c.1284G>A, p.M428I) in the patient as well as in two affected male family members that became known during the sequencing process (Fig. 1). All maternal alleles showed heterozygosity and healthy males were hemizygous wild type (Supplementary Fig. 1A), confirming complete segregation of the c.1284G>A mutation with disease in agreement with X-linked inheritance. Exome and Sanger sequencing of ATP6AP1 in a cohort of unsolved male patients with abnormal protein glycosylation revealed additional mutations in eight patients from five families (Table 1, Supplementary Fig. 1B). Patient 2.1 showed a c.431T>C (p.L144P) missense mutation, heterozygous in the mother and absent from the father and a healthy sister. An additional hemizygous missense mutation (c.1036G>A, p.E346K) was identified by Sanger sequencing in three non-related male sib pairs (families 3–5). Fathers carried wild-type alleles and mothers were heterozygous for the c.1036G>A variant, in agreement with X-linked inheritance. Exome sequencing of patient 6.1 revealed a c. 938A>G (p.Y313C) missense mutation.

All four missense mutations (L144P, Y313C, E346K and M428I) affect amino acids that are highly conserved down to fruitfly, tetraodon and frog (Supplementary Fig. 1C). Ac45 homologues in more distantly related species could not readily be retrieved by use of standard BLAST searches. L144P is located in the N-terminal domain, while Y313C, E346K and M428I are located in the processed C-terminal domain of Ac45 (Fig. 1b).

Clinical phenotype of Ac45 deficiency. The clinical phenotype of Ac45 deficiency.

In vertebrates, Ac45 is ubiquitously expressed with the V-ATPase assembly factor Voa1 mutant yeast with the processed C-terminal domain of Ac45 (Fig. 1b). The Ac45 protein is involved in membrane trafficking and Ca^{2+}-dependent membrane fusion. V-ATPase assembly has been extensively studied in yeast, where Vma12 and Vma22 cooperate in the assembly of the V_0 domain in the endoplasmic reticulum (ER) membrane. Additionally, yeast Voa1 has been established as an ER-localized V_0-assembly factor. In 2008, however, no human orthologue has been identified so far. In human, V-ATPase assembly is hardly studied, and no yeast orthologue of human Ac45 has thus far been identified.

In this study, we describe a novel ATP6AP1-linked immuno-deficiency and identified disease mutations in ATP6AP1 in 11 male patients with abnormal protein glycosylation. Yeast V-ATPase assembly factor Voa1 was predicted to be homologous to Ac45, which was confirmed by functional complementation of Voa1 mutant yeast with the processed C-terminal domain of Ac45. Identification of different Ac45 protein isoforms in human brain, liver and B cells indicated the presence of tissue-specific regulation of organelle acidification.
Liver biopsy findings. Liver biopsy was performed in six patients and was (near)-normal for patients 1.3 and 2 (with the substitutions p.M428I and p.L144P, respectively), but revealed steatosis, fibrosis and even micronodular cirrhosis in patients with the p.E346K mutation (Table 1, Supplementary Fig. 2). When performed, radiography showed no signs of osteopetrosis (ref. 29). When performed, radiography showed no signs of osteopetrosis (ref. 29).

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The index family (substitution p.M428I) presented with a milder disease course (oldest patient 34 years of age). All three patients in this kindred presented with sensorineural hearing loss of various extents and hypoplasia. The grandmother also presented deafness at older age. The vision abnormality was of various extents and hyperopia. The grandmother also presented deafness at older age. The vision abnormality was of various extents and hyperopia.

Table 1 | ATP6AP1-deficient patients: genetic, clinical and laboratory data.

<table>
<thead>
<tr>
<th>Family</th>
<th>Ethnicity</th>
<th>Consanguinity</th>
<th>Mutation protein</th>
<th>Laboratory abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Caucasian</td>
<td>Yes</td>
<td>p.Met428Ile (M428I)</td>
<td>Neutropenia, Leucopenia</td>
</tr>
<tr>
<td>2</td>
<td>Druze</td>
<td>Yes</td>
<td>p.Leu144Pro (L144P)</td>
<td>Neutropenia, Leucopenia</td>
</tr>
<tr>
<td>3</td>
<td>Caucasian</td>
<td>Yes</td>
<td>p.Tyr313Cys (Y313C)</td>
<td>Neutropenia, Leucopenia</td>
</tr>
<tr>
<td>4</td>
<td>Tunesian</td>
<td>Yes</td>
<td>p.Glu346Lys (E346K)</td>
<td>Neutropenia, Leucopenia</td>
</tr>
<tr>
<td>5</td>
<td>Irish</td>
<td>Yes</td>
<td>p.Glu346Lys (E346K)</td>
<td>Neutropenia, Leucopenia</td>
</tr>
<tr>
<td>6</td>
<td>Irish</td>
<td>Yes</td>
<td>p.Tyr313Cys (Y313C)</td>
<td>Neutropenia, Leucopenia</td>
</tr>
</tbody>
</table>

IVIG, intravenous immunoglobulin; ND, no data; m, months; wks, weeks; y, year; N ~ O, borderline abnormal O-glycosylation.

The index family (substitution p.M428I) presented with a milder disease course (oldest patient 34 years of age). All three patients in this kindred presented with sensorineural hearing loss of various extents and hypoplasia. The grandmother also presented deafness at older age. The vision abnormality was of various extents and hyperopia. The grandmother also presented deafness at older age. The vision abnormality was of various extents and hyperopia.
patients tested (3.1 and 3.2). Mass spectrometry of total serum N-glycans revealed minor accumulations of truncated glycans (Fig. 3b) and no overlapping glycan signature could be regarded as specific for Ac45 deficiency. Mass spectrometric analysis of isolated transferrin revealed a clear accumulation of similar types of truncated glycans lacking galactose and sialic acid in all patients (Fig. 3c, Supplementary Fig. 3).

Differential processing of Ac45 in liver, brain and B cells. We studied ATP6AP1 expression in human fetal and adult tissues. Both in fetal (data not shown) and adult tissues, the highest Ac45 mRNA expression was found in brain and the lowest expression level in liver and duodenum (Supplementary Fig. 4). To study Ac45 expression at the protein level, we performed western blot analysis of mouse cortex, human brain and liver, and human B cells using an Ac45 antibody directed to the C-terminal half of mouse Ac45. Ac45 is synthesized as a 62-kDa precursor protein (intact-Ac45) that in neuronal and neuroendocrine cells is subsequently processed to its ~40-kDa cleaved form (cleaved-Ac45 (refs 12,13,15,30), Fig. 4a). In mouse and human brain, most Ac45 protein was present in its cleaved ~40-kDa form, with human Ac45 migrating slightly faster than its mouse counterpart (Fig. 4b, lanes 1 and 3). Furthermore, and in-line with earlier studies in Xenopus neuroendocrine cells31, these proteins were N-glycosylated as shown by their sensitivity towards endoglycosidase PNGaseF (Fig. 4b, lanes 2 and 4). These results are in agreement with the slightly lower molecular mass of human Ac45 as compared with mouse Ac45 and the presence of one extra N-glycan on mouse Ac45. In addition, in human brain a thus far unknown 50-kDa form was observed. In human and mouse liver, considerable Ac45 protein expression was observed, predominantly as the 62-kDa intact proteoform. Under the conditions used, this band was insensitive to PNGaseF treatment (Fig. 4b, lanes 5 and 6). Western blot analysis of primary B-cell isolates as well as B-cell lines (data not shown) revealed Ac45 as an ~50-kDa protein isoform (Fig. 4c). Analysis of Ac45 protein expression in patient liver biopsy material revealed a strong reduction in the expression of the ~62- and ~40-kDa Ac45 variants and an additional ~50-kDa protein was observed (Fig. 4d).

Subsequently, we performed newly synthesized protein labeling with 35S methionine in immortalized human hepatocytes
Human Ac45 is orthologous to yeast Voa1 and Big1. Orthologs of Ac45 were readily identified by BLAST among the metazoan, including nematodes like Caenorhabditis elegans, but not outside of that taxon, leading to speculations about a role of Ac45 in specialized and complex vacuolar systems in multicellular organisms. Nevertheless, the degree of sequence identity between vertebrate and invertebrate members of the protein family is relatively low, suggesting a high rate of sequence evolution as an alternative explanation for the inability to detect non-metazoan homologues. Using orthology prediction at the level of sequence profiles, we detected two S. cerevisiae Ac45 homologues: Voa1 and Big1. Voa1’s C-terminal transmembrane helix is significantly similar to the C terminus of Ac45 (E = 9.1e-5) (Fig. 5a), while the sequence similarity of Ac45 to Big1 (E = 3.6e-10) is mainly restricted to the N-terminal ~250 amino-acid residues of Ac45 and therewith coincides with the part of Ac45 that is proteolytically cleaved by furin. No significant sequence similarity could be detected in the dotted lines, or for the comparison of the C-terminal helix of Big1 with Ac45. Both proteins are located in the ER membrane, where Voa1 has been implicated in assembly of the trans-Golgi network (TGN) or components of the endosomal system (Fig. 4f, Supplementary Fig. 6).

Processed Ac45 functions in place of Voa1 in S. cerevisiae. In yeast, Vma21 and Voa1 are assembly factors of the V-ATPase V₀ domain in the ER membrane. Both are retained in the ER via a C-terminal dilysine motif. When this motif is mutated to diglutamine, the resulting Vma21QQ or Voa1QQ protein is mislocalized to the vacuole with concomitant reduction in V-ATPase assembly and activity. The effect on V-ATPase assembly is cumulative, becoming most apparent when Voa1 is absent (voa1::H) or Voa1QQ is expressed in vma21QQ cells. Yeast lacking functional V-ATPase have a characteristic growth phenotype: they are unable to grow on medium buffered to pH 7.5, or medium containing elevated levels of calcium, or a combination of the two stresses. Reduced V-ATPase function can be detected by reduced growth under any of these conditions. A growth assay on rich medium supplemented with 100 mM CaCl₂ was used to assess the ability of human Ac45 to substitute for Voa1 (Fig. 5b,c). Full-length or processed Ac45 proteins with or without a dilysine motif (KKNN) appended to the C terminus were expressed in a yeast background having either a deletion or wild type of Voa1::H vma21QQ or Voa1QQ protein is expressed in vma21QQ cells. Yeast lacking functional V-ATPase have a characteristic growth phenotype: they are unable to grow on medium buffered to pH 7.5, or medium containing elevated levels of calcium, or a combination of the two stresses. Reduced V-ATPase function can be detected by reduced growth under any of these conditions. A growth assay on rich medium supplemented with 100 mM CaCl₂ was used to assess the ability of human Ac45 to substitute for Voa1 (Fig. 5b,c). Full-length or processed Ac45 proteins with or without a dilysine motif (KKNN) appended to the C terminus were expressed in a yeast background having either a deletion or wild type of Voa1::H vma21QQ strain. Cells expressing full-length Ac45 grew poorly, comparable to cells
transformed with empty vector or cells expressing Voa1QQ. Adding a dilysine motif to full-length Ac45 did not significantly improve growth. Conceivably, Ac45 function is dependent on proper processing of the protein, which might not be accomplished in yeast. Therefore, simulating a processed Ac45 protein\textsuperscript{12,14,15}, the C-terminal half of Ac45 was expressed. This processed Ac45 was able to function in place of Voa1, but only when expressed with a dilysine motif (cleaved-Ac45-KKNN in Fig. 5c). By complementation, cleaved-Ac45-KKNN function is comparable to that of Voa1.
Figure 4 | Differential expression of the Ac45 protein in human brain, liver and B cells. (a) Schematic representation of the human Ac45 protein. CS, furin proteolytic cleavage site; SP, signal peptide; TM, transmembrane domain; † represent predicted N-glycan structures, whereas the structures shown in black (†) are the experimentally confirmed glycans. (b) Western blot analysis of Ac45 in mouse cortex and in human brain and liver. Asterisk (*) indicates the deglycosylated form of cleaved-Ac45. Hash tags (#) indicate non-specific antibody reaction with PNGaseF present in the samples. (c) Western blot analysis of Ac45 in primary B cells from healthy controls in comparison with human liver. One of the two representative analyses is shown. (d) Western blot analysis of Ac45 in liver tissue homogenates of control and patient 4.2. GapdH was used as loading control. (e) Analysis of newly synthesized Ac45 in immortalized human hepatocytes (IHH). Cells were transfected with Ac45 construct, pulsed for a 30-min period with 35S, and Ac45 was immunoprecipitated and analysed by SDS–PAGE. Cells were treated with or without tunicamycin during the 30-min pulse (left panel). Immunoprecipitated Ac45 protein was treated with or without Endo H or PNGaseF (right panel). Note during the 30-min pulse period, the presence of a minor portion of newly synthesized pre-intact-Ac45 protein is still in its unglycosylated proform and containing the signal peptide for translocation over the ER membrane. (f) IHH cells were stained with anti-Ac45 antibody (green) and antibodies against various organelle markers (magenta). Nuclear staining is shown in blue (DAPI). Co-localization is indicated by a white colour in the merged channel. The graph shows the fluorescent intensity profile along the cross-section indicated. Scale bar represents 10 μm. Staining for Sec31 is shown as example, other organelle markers are shown in Supplementary Fig. 5.
Figure 5 | Identification of Voa1 as the yeast ortholog of human Ac45. (a) Overview of the regions of Ac45 that are homologous to the yeast proteins Voa1 and Big1 and an alignment of Ac45's and Voa1's C-terminal transmembrane helices (in blue, based on TMHMM63) and their flanking amino acids. Ac45 and Voa1 are separated by a sequence logo representation of this region among all the homologs that could be detected using JACKHMMER64. A pattern in which the level of sequence conservation in the transmembrane helix peaks every 3–4 amino acids is indicated with arrows. (b) Schematic of Voa1 and Ac45 proteins expressed from centromere plasmids in voa1::H vma21QQ yeast24. Ac45 proteins are either full length (intact-Ac45) or processed (cleaved-Ac45), with (shown) or without KKNN appended to the natural C terminus. Numbers indicate amino-acid residues. Residues mutated in Ac45 are shown. (c) Cleaved-Ac45 can substitute for Voa1 when a C-terminal dilysine motif is present. The voa1::H vma21QQ strain was transformed with plasmids coding for the indicated proteins (HA-tagged, diagrammed in (b)), Voa1QQ denotes Voa1 with K262Q and K263Q mutations. (d) The Y313C or E346K mutation in cleaved-Ac45-KKNN reduces V-ATPase function while protein levels are unaffected. Serial dilution growth test of voa1::H vma21QQ yeast expressing the indicated proteins tagged with HA. Restrictive growth is on rich medium adjusted to pH 7.5 and supplemented with 60 mM CaCl2. Membrane proteins prepared from the same cultures used in the growth test were analysed by western blot using anti-HA antibody to detect Voa1, cleaved-Ac45-KKNN and its mutant forms (band locations marked on the right, molecular mass (kDa) is indicated on the left. (e) Voa1 and cleaved-Ac45 require a C-terminal dilysine motif for ER localization. Fluorescent microscopy of live yeast cells showing DAPI stained DNA, GFP, the merged image of both, and cells viewed by differential interference contrast (DIC) to locate the vacuole as apparent indentation. The indicated proteins are N-terminally tagged with HA-GFP and expressed in voa1::H vma21QQ yeast cells. Exposure times for GFP images of cleaved-Ac45 were 10 × longer than for Voa1 or Voa1QQ. Perinuclear GFP fluorescence indicates ER localization. Mutated and non-mutated cleaved-Ac45-KKNN show the same localization. See also Supplementary Fig. 7.
Growth assays were used next to measure the effect of three pathogenic Ac45 substitutions, Y313C, E346K or M428I (Fig. 5d, left panels). The mutations were introduced into cleaved-Ac45-KKNN and expressed in voa1::H vma21QQ yeast. Growth was tested on rich medium supplemented with 60 mM CaCl2 and buffered to pH 7.5. Cells expressing Y313C or E346K mutant protein showed a growth defect, while the E346K mutant most severely compromised, exhibiting reduced growth nearing that of yeast having no Voa1. The effect of the M428I substitution was less disruptive and appeared indistinguishable from non-mutated Ac45. To ascertain that the growth defect observed for the Y313C or E346K substitution was not the result of protein instability, membrane proteins from the cells used in the growth assay were examined by western blot (Fig. 5d, right panel). While Ac45 protein levels were lower than Voa1, levels for mutated Ac45 proteins were unchanged compared with non-mutated Ac45 protein. Therefore, reduced V-ATPase function observed for the Y313C or E346K mutation cannot be ascribed to decreased protein abundance.

Since both Voa1 and processed Ac45 require a dileucine motif for function, it is expected that, like Voa1, processed Ac45-KKNN is retained in the ER membrane, while absence of the motif would result in mislocalization to the vacuole. This was tested using GFP-tagged proteins (Fig. 5e, Supplementary Fig. 8). Though GFP-tagging slightly reduced fitness of processed Ac45-KKNN on restrictive medium (data not shown), dileucine-dependent ER localization was verified. Together with growth assay results, these results indicate that the human and yeast proteins function about equally well in V0 V-ATPase assembly in the ER.

Discussion

Much has been learned about V-ATPase function and assembly by studies in yeast, however, studies in human have been very limited. The clinical symptoms resulting from Ac45 deficiency, mostly affecting the liver, immune system and brain, significantly differ from other known human genetic defects in various mostly affecting the liver, immune system and brain, significantly limited. The clinical symptoms resulting from Ac45 deficiency, Much has been learned about V-ATPase function and assembly.

The combination of clinical symptoms as observed in Ac45-deficient patients is currently poorly understood since research on the functional roles of Ac45 has mainly been focused on neuroendocrine cells and osteoclasts. Certain specific symptoms could be related to other V-ATPase defects or known forms of Ac45. for example, renal tubular acidosis or B-cell activation. In addition, electron microscopy of a liver biopsy of patient 31 suggested evidence for enhanced mitochondrial autophagy and muscle weakness with mildly elevated creatine kinase was found in some patients. This could suggest a partially overlapping disease mechanism with Vma21-deficient XMEA patients. Finally, in two of the patients, decreased enamelization of the teeth was reported, which could correspond with a recently reported role of V-ATPase-mediated acidification in enamelization. Thus, likely at least some of the symptoms are related to V-ATPase dysfunction, which is supported by our studies on V-ATPase restricting growth conditions in yeast.

Immune and liver dysfunction have not yet been reported in genetic defects of the V-ATPase, although liver disease was recently described for defects in V-ATPase assembly factors TEM199 and CCDC115 (refs 39,40). The question is why these systems are affected. Possible explanations could include the tissue-specific processing of Ac45 or the existence of additional functions of Ac45 beyond pH regulation via its effect on the V-ATPase. Our studies in human hepatocytes show that Ac45, in contrast to what was observed in neuroendocrine cells, mostly localizes to the early secretory pathway. This is in agreement with the presence of mostly intact-Ac45 carrying non-processed high-mannose glycans. Further studies are needed to elucidate the mechanisms driving differential Ac45 glycosylation and processing in brain, liver and immune cells, since tissue-specific forms of Ac45 could suggest a possible mechanism for the tissue-restricted disease symptoms in Ac45-deficient patients.

Thus far, the relationship of Ac45 with immune deficiency has remained unnoticed. In view of the reported multiple functions of Ac45 in, for example, pH regulation and membrane trafficking and fusion, many possible links exist. Acidification of phagolysosomes in, for example, macrophages is important for killing of internalized microorganisms, while antigen processing is also dependent on acidic pH. Our growth assay in yeast under conditions that are dependent on V-ATPase activity support the notion that the patients’ phenotypes could be related to aberrant acidification due to dysfunction of the V-ATPase. Membrane trafficking and fusion events have not only been linked to V-ATPase function but also to Ac45 (refs 18,43). These events are reported to be required for B-cell differentiation, antigen processing and antibody production. Thus, pathogenic mutations in ATP6AP1 might affect B-cell function at all these levels, resulting in decreased levels of immunoglobulins and recurrent infections in our patients. The observed hypoglycosylation on serum transferrin in our patients might indicate hypoglycosylation on other proteins as well. Several membrane-bound proteins such as CD19 and CD40 that are involved in B-cell activation are glycosylated, and antigen recognition and antibody production by B cells require fucosylated IgG-BCR. A glycosylation defect therefore may affect B-cell activation and thus antibody production. To find out which processes, that is, glycosylation, vesicular trafficking and fusion, or pH regulation are mainly affecting antibody production by a defective accessory subunit of the V-ATPase, further studies are required.

Previous studies have described an important role for Ac45 in intraorganellar pH regulation and membrane trafficking. Identification of processed Ac45 as the functional ortholog of yeast V-ATPase assembly factor Voa1 only when the KKNN ER retention signal is present, indicates the importance of ER localization for its function in yeast, and provides a valuable model to further dissect the different functions and functional domains of Ac45. As the human ortholog lacks this dileucine motif, other mechanisms might account for retention of Ac45 to the ER of specific cell types such as liver cells. Our observation in liver cells that the Ac45 protein is mostly present in its unprocessed form, which in neuroendocrine cells appears to implicate ER localization, combined with its observed steady-state localization in the early secretory pathway (ER, ERGIC) in hepatocytes, suggests that differential proteolytic processing might represent such a mechanism.
In summary, the identification of tissue-specific proteolytic processing of Ac45, and the availability of Voα1 mutant yeast as a valuable model to further dissect the individual functions of Ac45 will facilitate future research to understand the functional roles and isoforms of Ac45 in the immune system, liver, muscle and brain and its relation to the V-ATPase in human. Screening for abnormal protein glycosylation in plasma of patients with hepatopathy and immune dysfunction with or without neurological symptoms provides a rapid way to identify additional individuals with ATP6AP1 deficiency.

Methods

Patients and glycosylation studies. Blood and fibroblasts of patients (clinical information in Table 1) were obtained for diagnostics of inborn errors of metabolism. Blood space was received from patients and reference genome physicians. Isoelectric focusing of serum transferrin for analysis of protein N-glycosylation defects and of serum apolipoprotein CIII for analysis of mucin type O-glycosylation defects were carried out as described before49. Plasma N-glycan profiling was performed by MALDI linear ion trap mass spectrometry as described45 using 10 μl of plasma. Briefly, serum was treated with PNGaseF; free N-glycans were permethylated, extracted and dried, purified and spotted onto a MALDI plate. Samples were dried and measured on a linear ion trap mass spectrometer. High resolution mass spectrometry of intact serum transferrin was performed as described45. Briefly, transferrin was immunopurified from 10 μl serum using anti-transferrin Sepharose beads. The elution with glycine-HCl pH 2.7 was neutralized by Tris-HCl pH 9.0 and was directly available for injection onto the nanoLC-C8-chip of the QTOF. Transferrin was eluted from the chip in a 10 min gradient of H2O and Acetonitrile, 0.1% formic acid. Charge distribution raw data were deconvoluted by Mass Hunter software to reconstructed mass spectra51.

Next-generation sequencing. Genomic DNA was extracted from patient fibroblast according to the manufacturer’s protocol using a QIAamp Mini kit (Qiagen, Hilden, Germany), and was checked for DNA integrity on agarose gels. Next-generation sequencing and analysis was performed as described45. In brief, exon enrichment was performed using the SureSelect Human All Exon 50 Mb Kit (Agilent, Santa Clara, CA, USA). Colour barcodes were iteratively mapped to the hg19 genome with the SOLiD LifeScope software version 2.1. Called variants and indels were annotated using an in-house annotation pipeline25,27 and common variants were filtered out based on a frequency of >0.5% in dbSNP (137) and a frequency of >0.3% in our in-house database of >1,300 exomes. Quality criteria were applied to filter variant calling with <5 variant reads and <20% contamination. Furthermore, synonymous variants, dominant intronic, intergenic and UTR variants were excluded. Raw data of candidate variants were inspected using the Integrative Genomic Viewer software (IGV browser) version 2.3.14 (2013) (ref. 54) (http://www.broadinstitute.org/igv/download). The putative consequences of the mutations found in the Ac45 protein were predicted using Sift (2009) (http://sift.jcvi.org/www/SIFTaligned_segs_submit.html), Mutation taster, (2014) (http://www.mutationtaster.org/) and Polyphen-2 (2012) (http://genetics.bwh.harvard.edu/pph2/) prediction programs.

Sanger sequencing. Genomic DNA was extracted from fibroblast pellets or white blood cells from 10 patients and available family members. Primers (Supplementary Table 4) were designed to amplify the 10 exons of ATP6AP1 (GenBank accession number NM_001183.4), including at least 50 bp of the 5’- and 3’-untranslated regions. Standard PCR reactions were based on 1 μl DNA and 0.2 μl Platinum Taq polymerase (Invitrogen) in a total volume of 25 μl. Standard reaction conditions were 10 min at 95°C, then 35 cycles of 30 s at 95°C, 30 s at 60°C and 1 min at 72°C. The reaction was completed with a final elongation of 7 min at 72°C. From the PCR product, the Terminator Ready reaction cycle sequencing kit v3.1 (Applied Biosystems) was used. Analysis of the results was performed on an ABI3100 Avant (Applied Biosystems).

ATP6AP1 gene expression profiling in human tissues by qPCR. Total RNA from different human adult and fetal tissues was ordered from Stratatogene (Amsterdam, The Netherlands). All fetal tissues are from 20- or 21-week-old embryos after gestation. RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocols. To remove residual traces of genomic DNA, the RNA was treated with DNase I (Invitrogen, Leek, The Netherlands) while bound to the RNA binding column. The integrity, concentration and purity of the RNA were assessed using agarose gel electrophoresis and spectrometry. Of all tissues, 5 μg of total RNA was transcribed into cDNA by using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s protocol. cDNA was purified by using the Nucleospin extract II kit (Macherey-Nagel) according to the manufacturer’s protocol. Quantitative PCR quantifications were performed in on the equivalent of 12.5 ng total RNA input using the Sensifast SYBR no ROX qPCR kit (Bioneer) and a Rotor-GenETM 6000 real-time analyzer (Qiagen). qPCR program used was (2 min 95°C (5 s 95°C, 10 s 60°C or 65°C and 15 s 72°C) × 40 cycles). Two pairs of intra-spanning ATP6AP1 primers were used. Primers are listed in Supplementary Table 4. As reference transcripts, GUSB and PPIB were used. qPCR data were analysed by using comparative quantitation and the relative expression of the genes of interest calculated by equalizing the lowest Ct value to 1. The normalization factor for the reference genes was determined using the GeNORM program (medgen.ugent.be/genorm) and used to normalize the Q-values. Individual experiments were performed in triplicate.

Biochemical studies in immortalized human hepatocytes. IHH53 were cultured in gelatin-coated culture flasks in Williams medium E supplemented with 10% FCS, 0.022 μM insulin and 0.045 μM dexamethasone. Culturing was done at 37°C under an atmosphere of 5% CO2. IHH cell cultures were tested negative for mycoplasma. For Ac45 expression, IHH cells were transfected with a hAsAc45/pCgendNA3 construct using Lipofectamine LTX (Invitrogen) according to manufacturers’ guidelines.

For immunofluorescence assays, cells were cultured on gelatin-coated cover slips for 3 days and fixed for 1 h by 4% paraformaldehyde in PBS at room temperature. After blocking of residual parafomaldehyde with 50 mM NH4Cl in PBS, cells were permeabilized using 0.1% Triton-X100 and 0.2% polyvinylidene difluoride membrane. After blocking in 5% milk in PBS + 1% Tween-20, the membrane was incubated overnight at 4°C with primary antibody rabbit anti-mouse IgG polyclonal antibody #49 antiserum (directed against AS2T-283 and L443-1457 of mouse Ac45, kindly provided by Dr. J. Creemers, Catholic University, Leuven, Belgium) at a dilution of 1:20,000 in blocking buffer. Goat-anti-rabbit-HRP secondary antibody (Dako, PO448) at a dilution of 1:5,000 in 2.5% milk in PBS-1% Tween-20 were used for ECL detection. To check for protein loading, the membrane was incubated with mouse anti-GAPDH monoclonal antibody (Ab8245-100, Abcam) at a dilution of 1:2,000 in 3% BSA in PBS/0.1% Tween-20 for 1 h at room temperature. The incubated secondary antibody (Goat-antimouse-HRP, Dako PO447) was for 1 h at room temperature at a dilution of 1:5,000 in 2.5% milk in PBS-1% Tween-20. Chemoluminescent signals was detected using ECL (Pierce).

In summary, the identification of tissue-specific proteolytic processing of Ac45, and the availability of Voα1 mutant yeast as a valuable model to further dissect the individual functions of Ac45 will facilitate future research to understand the functional roles and isoforms of Ac45 in the immune system, liver, muscle and brain and its relation to the V-ATPase in human. Screening for abnormal protein glycosylation in plasma of patients with hepatopathy and immune dysfunction with or without neurological symptoms provides a rapid way to identify additional individuals with ATP6AP1 deficiency.
Newly synthesized protein labelling experiments were performed to study the synthesis and processing of newly synthesized proteins. Cross sections were used to visualize the localization of newly synthesized proteins. Relative fluorescence intensities were calculated over a 10-minute incubation period. The highest measured value was set to 100.

Bio-informatics studies. For sequence-profile-based homology searching, we used HHpred to search for homologs of Voa1 amino acids I218–I261. Using HHpred, the expectation values (E-values) were obtained via homology detection using HHPred. The sequence profile-based homology searching was performed using HHPred. Similarity searches were performed using the sequence profiles of Voa1.

Transmission electron microscopy. The tissue cylinder was immediately immersed in cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, post-fixed in 1% osmium tetroxide for 1 h, dehydrated through ethanol series and embedded in epoxy resin. Ultrathin sections (60 nm) were cut with diamond knives and examined with a transmission electron microscope.

Live cell imaging of yeast cells. Yeast cells expressing GFP-tagged proteins were grown overnight in SD-Ura, diluted to 0.2 OD600 per ml in SD-Ura containing 2.5 mg ml−1 DAPI (Sigma) and grown until 0.4 OD600 per ml. Cells were collected by centrifugation and incubated in the same buffer but without milk. Before imaging (Odyssey Fc Imager, LI-COR), a final wash was done in 10 mM Tris-HCl pH 7.5, 150 mM NaCl.

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files.

References

Yeast strain and plasmids. Standard molecular biology protocols for E. coli and yeast manipulations were followed. The N-terminal half of ac45 (ref. 24) was derived from the strain VOA1/a(DE3) (ref. 25) and expressed in the yeast strain 35273/MRY5 (ref. 26) and the plasmid pMR1214 was derived from the plasmid pMR1213 by inserting a 5′ UTR flanking sequence.
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