V₁ and V₀ Domains of the Human H⁺-ATPase Are Linked by an Interaction between the G and a Subunits*

Elizabeth E. Norgett‡, Katherine J. Borthwick‡, Rafia S. Al-Lamki‡, Ya Su‡, Annabel N. Smith‡, and Fiona E. Karet‡†
From the ‡Department of Medical Genetics, ¶Department of Medicine and †Division of Renal Medicine, University of Cambridge, Cambridge CB2 0XY, United Kingdom

The specialized H⁺-ATPases found in the inner ear and acid-handling cells in the renal collecting duct differ from those at other sites, as they contain tissue-specific subunits, such as a₄ and B₁, and in the kidney, C₂, d₂, and G₃ as well. These subunits replace the ubiquitously expressed forms. Previously, we have shown that, in major organs of both mouse and man, G₃ subunit expression is limited to the kidney. Here we have shown widespread transcription of murine G₃ in specific segments of microdissected nephron, and demonstrated additional G₃ expression in epithelial fragments from human inner ear. We raised a polyclonal G₃-specific antibody, which specifically detects G₃ from human, mouse, and rat kidney lysates, and displays no cross-reactivity with G₁ or G₂. However, immunolocalization using this antibody on human and mouse kidney sections was unachievable, suggesting epitope masking. Phage display analysis and subsequent enzyme-linked immunosorbent assay, using the G₃ antibody epitope peptide as bait, identified a possible interaction between the G₃ subunit and the a₄ subunit of the H⁺-ATPase. This interaction was verified by successfully using purified, immobilized full-length G₃ to pull down the a₄ subunit from human kidney membrane preparations. This confirms that a₄ and G₃ are component subunits of the same proton pump and explains the observed epitope masking. This interaction was also found to be a more general feature of human H⁺-ATPases, as similar G₁/a₁, G₃/a₁, and G₁/a₄ interactions were also demonstrated. These interactions represent a novel link between the V₁ and V₀ domains in man, which is known to be required for H⁺-ATPase assembly and regulation.

The vacuolar-type H⁺-ATPases are ubiquitous in nature and play an essential role in regulating proton flux at a number of sites, both intra- and extracellular. For example, intracellular acidification of various organelles, such as endosomes and lysosomes, is a prerequisite for their normal function. Although they are evolutionarily and structurally related to the mitochondrial F-ATPases that synthesize ATP, the H⁺-ATPases found in mammalian systems have lost the capacity to synthesize ATP and instead function solely as rotary motors, using ATP hydrolysis to move protons across membranes (1).

The general structure of H⁺-ATPases has been largely modeled from yeast. It is similar across the wide variety of tissues and cell types in which they are found, but there are subtle differences between individual cell types. Each proton pump consists of at least 13 subunits organized into two distinct domains. In the current structural model of the H⁺-ATPase, the peripheral V₁ (hydrolytic) domain contains 3A, 3B, one each of C, D, E, F, and H, and two G subunits. This is loosely linked to the membrane-anchored V₀ domain by two stalks; the central stalk consists of the D and F subunits, and the peripheral stalk contains the C, E, G, and H subunits together with the N terminus of the a subunit (1–4). The a subunit is present as a single copy in the V₀ domain along with single copies of the d and e subunits and a ring structure comprising multiple numbers of c and c’ (in yeast cells) and a single c” subunit (1, 3, 5, 6).

In higher organisms, it is generally accepted that ubiquitously expressed H⁺-ATPases have been largely modeled from yeast. It is similar across the wide variety of tissues and cell types in which they are found, but there are subtle differences between individual cell types. Each proton pump consists of at least 13 subunits organized into two distinct domains. In the current structural model of the H⁺-ATPase, the peripheral V₁ (hydrolytic) domain contains 3A, 3B, one each of C, D, E, F, and H, and two G subunits. This is loosely linked to the membrane-anchored V₀ domain by two stalks; the central stalk consists of the D and F subunits, and the peripheral stalk contains the C, E, G, and H subunits together with the N terminus of the a subunit (1–4). The a subunit is present as a single copy in the V₀ domain along with single copies of the d and e subunits and a ring structure comprising multiple numbers of c and c’ (in yeast cells) and a single c” subunit (1, 3, 5, 6).

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demonstrated that both genes are expressed in epithelia within the inner ear (11, 12).

Preliminary expression studies of the other three subunits showed that, in the major organs in man, G3 subunit mRNA is only found in kidney, d2 is found in kidney, lung, and in osteoclasts in bone, whereas C2 is present in kidney and placenta (9, 13). G3 expression in mouse has been localized to intercalated cells of the cortical collecting duct (14). Here we have described further characterization of the human G3 subunit, which we have demonstrated is also expressed in the inner ear and which interacts within the pump complex with the a1 and a4 subunits. We have also shown that the ubiquitously expressed G1 subunit is able to interact with a1 and a4.

**EXPERIMENTAL PROCEDURES**

**Human Tissues**—With ethical approval from Addenbrooke’s Hospital Local Research Ethics Committee, samples of normal human renal tissue were obtained from nephrectomy specimens removed for renal tumors. Testis and epididymis were similarly recovered from the discarded normal portions of resection specimens. Inner ear epithelia were obtained during routine surgical operations where the integrity of the cochlea was not required. Tissues were stored at −80 °C until required.

**Protein Extraction**—Membrane and cytosolic protein fractions were prepared as described previously from human, mouse, and rat kidney and also mouse brain and were stored at −80 °C until use (15).

**Generation and Characterization of Anti-G3 Polyclonal Antibodies**—A polyclonal antibody was raised against two peptides present in G3, IDQYRMQRDKEFRLKQ (peptide 1, mouse, and rat kidney and also mouse brain and were stored at 80 °C until required.

**Generation and Characterization of Anti-G3 Polyclonal Antibodies**—A polyclonal antibody was raised against two peptides present in G3, IDQYRMQRDKEFRLKQ (peptide 1, amino acids 44–59), and LGKIQELNGHYNKYMES (peptide 2, amino acids 77–93). The peptides were linked to keyhole limpet hemocyanin and used to immunize a single sheep. The resulting serum was affinity-purified against the immunizing peptides, using a SulfoLink\textregistered column (Pierce) according to standard methods. The specificity of both unpurified and affinity-purified antisera was assessed by enzyme-linked immunosorbent assay using standard methods.

40 μg of each protein lysate were loaded on a 15% SDS-polyacrylamide gel, and Western blot was carried out following standard protocols. Membranes were blocked in phosphate-buffered saline (PBS)\(^2\) containing 5% nonfat milk and 0.05% Tween 20 and probed with a 1:200 dilution of affinity-purified anti-G3 antibody followed by a 1:5000 dilution of horseradish peroxidase-conjugated donkey anti-sheep antibody (Abcam) before incubation with ECL-Plus reagent (Amersham Biosciences) and visualization.

**Cloning of G3 into the minipRSETA-mac Bacterial Expression Vector**—Full-length human G3 was PCR-amplified from kidney cDNA using primers 5′-AATCAGCAGTACAACGCAAGCAGTCTCAGGGGATC-3′ and 5′-CTCCAGCCTTGGAAGA-ACTGTGATGTACATTTAGTTG-3′ to incorporate a 5′ PstI site and 3′ HindIII site, respectively. Full-length human G1 was PCR-amplified from kidney cDNA using primers 5′-CGGGTACCCTATGCTAGTCTC-3′ and 5′-CTCCAGCCTTGGAAGA-ACTGTGATGTACATTTAGTTG-3′, which incorporate a 5′ KpnI site and 3′ HindIII site, respectively. These sites were used to clone gel-purified PCR products into a modified pRSETA-mac vector (gift from Dr. Jane Clark, University of Cambridge). The plasmids were transformed into BL21 electrocompetent Escherichia coli, and clones containing the fusion constructs were identified using colony PCR and restriction enzyme digestion; the correct sequence of the inserts was verified using ABI Prism\textsuperscript{®} BigDye\textsuperscript{TM} Terminator cycle sequencing (Applied Biosciences).

**Expression, Purification, and Refolding of Full-Length G3-RGS(His)\(_d\) and G1-RGS(His)\(_o\)**—Following optimization of the expression conditions, 50 ml of LB broth (containing ampicillin at a final concentration of 0.1 mg/ml) were inoculated with bacterial cells containing either the RGS(His)\(_d\)-G3 or RGS(His)\(_o\)-G1 fusion construct. After initial incubation at 37 °C overnight, this culture was diluted 1:50 in a total volume of 2 liters and incubated at 37 °C to an A\(_{600}\) of 0.5–0.8. Expression of G3 or G1 was induced by the addition of isopropl 1-thio-β-D-galactopyranoside at a final concentration of 0.2 mM, and the cultures were incubated at 37 °C for 3 h. Bacterial cells were pelleted by centrifugation; the pellets were then lysed in B-per bacterial protein extraction reagent (Pierce) with one complete EDA-free protease inhibitor mixture tablet (Roche Applied Science) and benzonase nuclease (Novagen) (25 units/ml) for 1 h at room temperature with rotary shaking. Cell debris was pelleted by centrifugation, and the expressed epitope-tagged G protein was purified under denaturing conditions.

To achieve this, RGS(His)\(_d\)-G1 and RGS(His)\(_o\)-G3 cell debris was incubated at 4 °C for 30 min with PBS containing 8 mM urea, 300 mM NaCl, and 10 mM imidazole. Following centrifugation at 35,000 × g for 30 min, the supernatant containing the denatured insoluble proteins was removed and incubated with equilibrated nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen) for 1 h at 4 °C. Unbound protein was washed away with 200 ml of PBS containing 8 mM urea, 300 mM NaCl, and 30 mM imidazole. Immobilized, purified, epitope-tagged G protein was then refolded on the beads by repeatedly washing with 200 ml of PBS containing 300 mM NaCl, 30 mM imidazole, 5 mM β-mercaptoethanol, and decreasing concentrations of urea (8, 6, 4, 2, 1, 0.5, and 0.25 mM). Following a final 200-ml wash in PBS with 5 mM β-mercaptoethanol, purified G protein was eluted off the beads in PBS containing 500 mM imidazole and 5 mM β-mercaptoethanol. Protein was then dialyzed against PBS containing 5 mM β-mercaptoethanol, and purity was analyzed by SDS-PAGE. Soluble G protein was then rebound to Ni-NTA beads to carry out pulldowns.

**Pulldown of a4 from Human Kidney Using Immobilized G3 and G1**—Human kidney membrane protein preparation containing 500 μg of total protein was incubated for 30 min at 4 °C

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\(^2\) The abbreviations used are: PBS, phosphate-buffered saline; Ni-NTA, nickel-nitrilotriacetic acid; RT, reverse transcription; SSC, saline-sodium citrate.
in solubilization buffer containing 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 1.5% n-nonyl-β-D-glucopyranoside, and a protease inhibitor mixture tablet. Following centrifugation at 100,000 x g for 1 h, the supernatant containing the solubilized membrane proteins was removed and precleared by incubating with equilibrated Ni-NTA beads for 5 h at 4 °C. The precleared lysate was incubated with either equilibrated Ni-NTA-agarose or G1/G3-Ni-NTA overnight at 4 °C. Unbound proteins were washed away with buffer (pH 8) containing 50 mM NaH2PO4, 300 mM NaCl, and 20 mM imidazole. The Ni-NTA and G1/G3-Ni-NTA-agarose were each removed into 100 ml of Laemmli buffer and heated to 95 °C for 5 min. Boiled samples were centrifuged at 200,000 x g for 2 min, and the supernatant was removed for SDS-PAGE and Western blot analysis. Samples were loaded on 7.5 or 15% SDS-polyacrylamide gels, and Western blot was carried out following standard protocols. Membranes were blocked in PBS containing 5% nonfat milk (w/v), 0.05% Tween 20, and probed with either rabbit antiserum against a4 (1:1000) (8), goat anti-α1 antibody (1:250, clone C-16, Santa Cruz Biotechnology), or mouse anti-RGS(His)3 (Qiagen, 1:1000 dilution in 3% bovine serum albumin). Appropriate horseradish peroxidase-conjugated secondary antibodies were then used by incubation with ECL-plus reagent (Amersham Biosciences) and visualization.

Reverse Transcription (RT)-PCR—Total RNA was extracted from discrete structures within the inner ear and male genital tract using TRIzol reagent (Invitrogen). 2 μg of kidney RNA or the whole aliquot of inner ear RNA was treated with RNase-free DNase I (Promega) and then reverse-transcribed using Super RT (HT Biotechnology Ltd.) and oligo(dT)16 (New England BioLabs) as the primer according to the manufacturer’s protocol. cDNA was amplified using primers G3F2 (5’-ATGCAAGAGATAAAGAGTTTGAC-3’) and G3ExR3 (5’-CAGTGGCGATGTGATGTGATTGC-3’). These primers lie in exon 2 and the 3’ UTR of ATP6V1G3, respectively, and the expected product is 360 bp in size. To confirm successful reverse transcription, a 540-bp fragment of β-actin was amplified in parallel using primers 5’-GTGGGGCGCCC-TAGGCA-3’ and 5’-CTCTTGATGTGACGCACTTT-C-3’. In each experiment, sterile water was used as a template to provide a negative control. Reverse transcription reactions performed in the absence of enzyme provided a negative control sample for each tissue (data not shown). PCR products were visualized under UV light following electrophoresis on a 2% agarose gel containing ethidium bromide at a final concentration of 0.5 μg/ml.

Nephron Segment RT-PCR—To define the cell populations that contain G3, PCR was performed on cDNA isolated from discrete structures within the mouse nephron (gift from Dr. Carsten Wagner, University of Zurich, Switzerland) using primers MmG3F1 (5’-AAGGAAAGCTATGAGAGCAAG-3’) and MmG3R1 (5’-TCATACTAAACGCGCATCCAGGAG-3’). PCR products were visualized as above.

In Situ Hybridization and Immunofluorescence—Oligonucleotide probes corresponding to nucleotides 561–620 on the sense strand and nucleotides 620–561 on the antisense strand of human ATP6V1G3 (GenBank™ accession number NM_133262) were synthesized with a 5’ digoxigenin label (MWG Biotech). Probes were resuspended in RNase-free water, and dot blots were performed to ascertain the appropriate dilution factors. Frozen kidney was cut into 5-μm sections and immersed into PBS containing 4% paraformaldehyde for 5 min. Following two 5-min washes in PBS, the sections were incubated with PBS containing proteinase K (5 μg/ml) for 5 min and then with PBS containing glycine (2 mg/ml) for 5 min. Following 5 min of incubation in 2× saline-sodium citrate (SSC) buffer, sections underwent prehybridization at 37 °C for 1 h in hybridization buffer (1 mM Tris-HCl, pH 7.4, 2× SSC, 0.1 mM EDTA, 10% dextran sulfate, 1× Denhardt’s solution, and 50% formamide). Probes were then diluted in this buffer and hybridized to the sections overnight at 37 °C. The sections were washed in the following order: 5 min in 2× SSC at 37 °C; 3× 5 min in 0.2× SSC, 60% formamide at 37 °C; 2× 5 min in 2× SSC at room temperature; and then 5 min in in situ buffer (1 0.1 mM Tris, pH 7.4, 0.1 mM NaCl, 2 mM MgCl2, 0.05% Triton X-100) at room temperature. Non-specific antibody binding was blocked using in situ buffer containing 1% bovine serum albumin, and then anti-digoxigenin-AP antibody (Roche Applied Science) was added to the section (diluted 1:200 in in situ buffer containing 1% bovine serum albumin) and incubated for 2 h at room temperature. Following two 5-min washes in in situ buffer 1 and one 5-min wash in 0.1 mM Tris-HCl, pH 9.5, 0.1 mM NaCl, and 50 mM MgCl2 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma-Aldrich) was added to the section and incubated in the dark at room temperature to allow the color to develop. The reaction was stopped by immersion in water before counterstaining for the principal cell marker aquaporin 2 was performed. Sections were washed in PBS for 5 min, and then non-specific antibody binding was blocked using PBS containing 10% fetal calf serum for 45 min at room temperature. Anti-aquaporin-2 antibody (Chemicon) diluted 1:100 in PBS containing 10% fetal calf serum was added to the section and allowed to incubate at room temperature for 1 h. The sections were washed 3× 5 min in PBS, and then donkey anti-rabbit-Alexa Fluor 488 (Invitrogen) (diluted 1:200 in PBS containing 10% fetal calf serum) was added and allowed to incubate at room temperature for 1 h. Slides were washed three times for 5 min each in PBS, and then nuclear counterstain TO-PRO-3 iodide (Invitrogen) was added, diluted at 1:1000. After 5 min of incubation at room temperature, the sections were washed twice for 5 min each in PBS before being mounted in Glycergel (Dako).

RESULTS

Characterization of the Polyclonal G3 Antibody and Detection of G3 Protein in Human and Rodent Kidney—Having previously identified a novel form of the H+-ATPase G subunit, G3 (9), we first wished to investigate the localization of this protein within the kidney (9). To do this, we raised a polyclonal anti-G3 antibody using two peptides that are not homologously present within G1 or G2. An initial Western blot of human membrane and cytosolic kidney preparations using affinity-purified antiserum against both immunizing peptides detected a band of the appropriate size (14 kDa) in the membrane and, to a much lesser extent, in cytosol (Fig. 1, lanes 1 and 2). Competition
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FIGURE 1. Western blots were performed on human kidney cytosol (lanes 1, 3, 5, and 7) and human kidney membrane (lanes 2, 4, 6, and 8) using an affinity-purified antibody raised against two different G3 peptide sequences. A protein of appropriate size (14 kDa) can be seen in the membrane preparation (lane 2) and, to a lesser extent, in the cytosolic fraction (lane 1) when the membrane was probed with antibody affinity-purified against peptides 1 and 2. When the membrane was blocked with G3 peptide 1 (lanes 3 and 4), the 14-kDa protein is not detected (lane 4), whereas when the membrane was blocked with G3 peptide 2 (lanes 5 and 6), the 14-kDa protein can still be seen (lane 6), indicating that the 14-kDa protein is recognized by an antibody against G3 peptide 1. The secondary antibody alone does not recognize this 14-kDa protein (lanes 7 and 8).

FIGURE 2. The antibody raised against G3 peptide 1 detects a 14-kDa protein in human, mouse, and rat membrane fractions (lanes 2, 4, and 6) and to a lesser extent in cytosol (lanes 1, 3, and 5). Importantly, no band was seen from mouse brain preparations (lanes 7 and 8), demonstrating that the antibody does not cross-react with either the ubiquitously G1 protein or the brain-specific G2 protein. Size markers in kDa are indicated at the left of the image, and an arrow indicates the band corresponding to the G3 protein.

experiments, performed by incubating antiserum with a 3-fold excess of immunizing peptide prior to probing of the membrane, demonstrated that the immunogenic response was entirely to peptide 1 (Fig. 1, lane 4). Further experiments were therefore performed using affinity-purified antiserum against peptide 1 alone. By Western blot, this antibody specifically detects a 14-kDa protein in human, mouse, and rat kidney membrane fractions and, to a lesser extent, in cytosol (Fig. 2). Importantly, no band was seen from mouse brain preparations (lanes 7 and 8), demonstrating that the antibody does not cross-react with either the ubiquitously G1 protein or the brain-specific G2 protein. Although the antibody successfully and specifically labels G3 by Western blotting, positive immunostaining of kidney sections was not achievable using the G3-specific antibody, and epitope masking was suspected.

Phage Display Analysis—To investigate this possibility and to identify the interacting moiety, phage display was employed using the immunizing G3 peptide 1 as bait. Following three rounds of panning of the phage display peptide library with G3 peptide 1, the most common phage sequence observed was LPLTPLP. When this sequence was used to search the human genome data base using the “short, nearly exact match” BLAST algorithm, an exact match of phage peptide amino acids 2–7 was found to amino acids 85–90 of the H^+-ATPase subunit a4. This sequence is PLTPLP in a4 and PEPFP in a1. The specific interaction between the a4 7-mer phage peptide and G3 peptide 1 was confirmed by enzyme-linked immunosorbent assay (data not shown). We therefore hypothesized that the region of G3 represented by the immunizing peptide 1 directly interacts with the a4 subunit, and hence the epitope for the G3 antibody is masked by a4 in kidney sections.

Confirmation of G3-a4 Interaction and Analysis of Possible Interactions between Other a and G Subunits—To confirm the interaction between G3 and a4 and investigate the likelihood that other G and a subunits are also able to interact, full-length human G3 and G1 were separately cloned into a bacterial expression vector-incorporating sequence to create an N-terminal RGS(His)_n epitope tag. Epitope-tagged G proteins were successfully expressed in BL21 E. coli and refolded on Ni-NTA beads against a decreasing gradient of urea prior to elution using PBS containing imidazole and 5 mM β-mercaptoethanol. The soluble G protein was then rebound to Ni-NTA beads. Immobilized G3 was able to pull down the a4 subunit of the H^+-ATPase from solubilized human kidney membrane preparations (detected by Western blot), confirming the initial G-a interaction identified by phage display (Fig. 3A).

Following confirmation of the interaction between G3 and a4 subunits within the H^+-ATPase of human kidney, we wanted to investigate the possibility that the G-a interaction is a general phenomenon among different H^+-ATPases. The ubiquitously expressed a1 and G1 subunits are present in the kidney and inner ear, along with a4 and G3, which raises the possibility that they may be able to interact with each other and with the tissue-restricted subunits at these sites. Fig. 3 illustrates that this is the case; G3 was able to pull down the a1 subunit of the H^+-ATPase from solubilized human kidney membrane preparations (Fig. 3A). In parallel, immobilized G1 protein was able to pull down both a4 and a1 subunits from solubilized human kidney membrane preparations, confirming for the first time an interaction between V1 and V0 domains previously observed ex vivo only in yeast and Thermus thermophilus (Fig. 3B). We and others^3 have noted that all the available antibodies to a1 show a weak signal

3. U. Kornak, personal communication.
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FIGURE 4. A, expression of Atp6v1g3 is detected by RT-PCR in murine total kidney (lane 1), the proximal tubule S1 segment with glomerulus (lane 2), proximal tubule segments S1–S3 (lane 3), the thin loop of Henle (lane 4), cortical collecting duct (lane 5), and medulla (lane 6). The PCR controls, where no template was added to the reactions, were negative for both G3 and G3PDH (lanes 7, 11, and 14).

FIGURE 5. A, RT-PCR demonstrates that, in man, G3 is not detectable in testis (lane 1), epididymis (lane 2), endolymphatic sac (lane 3), and cochlea (lane 5) but is expressed in the vestibule of the inner ear (lane 4) and kidney (lane 6). B and C, G3PDH expression was demonstrated in the endolymphatic sac (lane 8), vestibule of the inner ear (lane 9), cochlea (lane 10), epididymis (lane 12), and testis (lane 13), illustrating the integrity of the cDNA template. The controls for the PCR, where no template was added to the reactions, were negative for both G3 and G3PDH (lanes 1, 11, and 14).

on Western blot, even in protein lysates where a1 should be present at high concentrations. Consequently, the signal from the pulldowns is less intense.

Expression of Human G3 in Inner Ear and Male Genital Tract—In major organs, transcription of G3 has previously been shown to be restricted to kidney (9). In this study, we assessed several other body tissues. Expression of G3 was additionally detected by RT-PCR in inner ear epithelia but not in the testis or epididymis (Fig. 4). This indicates an important species difference, because in a previous study in rat, both G3 and G1 proteins were detected in narrow and clear cells of the epididymis (16).

Nephron Segment RT-PCR—To begin to define the cell populations within the mammalian kidney that express G3, RT-PCR was performed on microdissected mouse nephrons (Fig. 5). Expression of murine G3 was detected throughout the post-glomerular tubule, except for the distal convoluted tubule.

Localization of G3 in Human Kidney by in Situ Hybridization—Consistent with these RT-PCR findings, in situ hybridization on human kidney sections revealed mRNA from Atp6v1g3 in a subset of tubular structures, but no staining was evident in glomeruli. The strongest signal was observed in a subset of tubules with collecting duct morphology, particularly in medulla. Importantly, no co-localization was seen between G3 and the principal cell marker aquaporin 2, confirming that G3-positive cells in this segment are intercalated cells (Fig. 6).

To extend these results further, immunoprecipitation using a murine polyclonal antibody against human G3, C2, and d2, respectively (9). Here, we have further characterized the G3 subunit, initially by use of a novel, G3-specific polyclonal antibody against human, mouse, and rat G3 protein that can be used for enzyme-linked immunosorbent assay and Western blotting. However, we were unable to obtain successful immunoprecipitation or immunostaining of human kidney, which prompted us to consider whether the epitope itself might be involved in subunit interactions. The subsequent studies presented here identified and confirmed an interaction between G3 and a4 in kidney, which is predicted to mask the epitope of the antibody. We have previously reported that a4 is also expressed in the inner ear and male reproductive tissue (12, 15), and here we show transcription of Atp6v1g3 in human inner ear epithelia but not in testis and epididymis. This therefore implies that G3 and a4 interact within the specialized proton pumps within the inner ear, but tissue confirmation is not presently possible.

This interaction is between two tissue-specific subunits, but importantly, we have gone on to identify similar interactions between the ubiquitously expressed G1 and a1 subunits. Previous studies have suggested that these ubiquitously expressed forms are found in the same pump complexes in non-special-
ized H\textsuperscript+-ATPases, such as those found in cellular organelles. For example, murine G1 and a1 have been co-precipitated with B2, suggesting that these ubiquitously expressed forms are present in the same pump complex (14). Additionally, we describe interactions between G3 and a1 and G1 and a4 forms.

In the current structural model of the H\textsuperscript+-ATPase, the peripheral V\textsubscript{1} domain is loosely linked to the membrane-anchored V\textsubscript{0} domain by two stalks, the central stalk and the peripheral stalk. The latter contains the C, E, G, and H subunits and the N terminus of the a subunit (1–4). Interactions within the V\textsubscript{1} domain between subunits E, G, and C have previously been demonstrated by cross-linking and in vitro pulldown techniques in yeast (17) and cow (18). To date, the only previous reports of interactions that connect the V\textsubscript{0} and V\textsubscript{1} domains within the peripheral stalk describe interactions in yeast between H and the N-terminal part of the a subunit, shown by two hybrid binding assays (19) and the ability of the a, G, and E subunits in *T. thermophiles* to form a stable complex (20). Additionally, cross-linking studies in yeast suggest links between the a and C subunits, but it is unclear whether this is a direct interaction *in vivo* (21).

The G-a interaction described here therefore provides further evidence that the N-terminal part of the a subunit is a component of the peripheral stalk in higher organisms, and this, in turn, supports the current structural model of the H\textsuperscript+-ATPase. It is notable that, unlike the other stalk components, two copies of subunit G are proposed to be present in the proton pump (18), with recombinant G protein spontaneously forming a dimer in solution (22). The presence of two copies of G within each pump may explain how the smallest pump subunit can interact with four other subunits.

Charsky *et al.* (23) have described the effects on the assembly and function of a number of point mutations and deletions in the yeast G subunit Vma10p. Mutation of amino acids Tyr-46, Lys-50, and Lys-55, which fall in the equivalent region of the protein as G3 peptide 1, shows lack of assembly of the V\textsubscript{1} sector with the V\textsubscript{0} sector and provides excellent supporting evidence for the interactions described here between the V\textsubscript{0} and V\textsubscript{1} subunits, which is vital for assembly of the H\textsuperscript+-ATPase. Although the H subunit is required for the activity of the H\textsuperscript+-ATPase, it is dispensable for assembly of the V\textsubscript{0} and V\textsubscript{1} domains, which further underscores the importance of the G-a interaction in the peripheral stalk described here (24). The G-a interaction may contribute to the regulatory mechanism for mammalian H\textsuperscript+-ATPase activity, as this has been shown in yeast to be attributable to the reversible dissociation of the V\textsubscript{0} and V\textsubscript{1} domains. This, in turn, is controlled by signals in the N-terminal part of the a subunit, the region we have shown interacts with the G subunit (25, 26).

Because expression of *ATP6V1G3* is limited to the kidney and inner ear, this gene is a good candidate to underlie inherited recessive distal renal tubular acidosis associated with deafness, a condition that we and others have previously shown to be attributable to mutations in *ATP6V0A4* and *ATP6V1B1*, which encode the a4 and B1 subunits of the H\textsuperscript+-ATPase, respectively (8, 10, 12). Genetic studies on families in which recessive disease is segregating and where linkage to *ATP6V0A4* and *ATP6V1B1* has been excluded have, to date, discounted the involvement of *ATP6V1G3* (9). However, results discussed here highlight the possibility that, when there are mutations in *ATP6V1G3*, they may be clinically undetectable because of the potential ability of other G subunits to bind to a4 and thus compensate for loss of G3 function.

In the apical membrane of human and rodent α-intercalated cells of the distal nephron, it is likely that G3, C2, and d2 would form part of the same pump as the tissue-limited B1 and a4 subunits. This is supported by the localization data presented here and also by previous studies in rodents (8, 14, 15, 27). In further support of this idea, co-localization of d2 and a4 has been demonstrated in human kidney by immunofluorescence (13), and in mouse, co-immunoprecipitation experiments have demonstrated that renal proton pumps containing the B1 subunit also contain the C2, G3, a4, and d2 subunits (14). Possible sites of different human G and a subunit interactions are summarized in Table 1.

The G1 and a4 interaction may occur *in vivo* at a number of possible sites. In rat, a1, a4, G1, and G3 have been shown to be present in narrow and clear cells of the epididymis and vas deferens (16), although we show here that G3 is not expressed in the human male reproductive tract. Therefore, it must be G1 rather than G3 that interacts with a1 and/or a4 in these tissues in man, highlighting the fact that species differences exist between rodent and man with regard to proton pump constituents in tissue-restricted specialized pumps (15, 16). Other examples of species difference between proton pump constituents have been noted previously, such as C2 expression in kidney and lung in mouse but only kidney and placenta in man (9, 14). It has also been demonstrated that a4 can be found at the brush border of murine and human proximal tubule cells (15). Consistent with an interaction in these cells, a4 has been co-immunoprecipitated with B2 in rodents, suggesting that it is found within the same H\textsuperscript+-ATPase complex as ubiquitous forms, such as G1 (14). G1 has also been reported to be present at the apical surface of α-intercalated cells in mouse kidney (although at lower levels than G3), where it is well established that a4 is a pump component (14, 27).

In terms of possible tissue co-localization for G3 with a1, we detected G3 expression along the nephron in segments known not to express a4 in man, and it is therefore likely that a1 contributes to H\textsuperscript+-ATPases at these sites. In rodents, both subunits have previously been localized to rat epididymis and vas deferens (along with G1 and a4); however co-immunoprecipitation experiments in mouse kidney failed to precipitate a1 with kidney-specific B1, a component of the same pump complex as G3; therefore, species differences are again likely to make extrapolation more difficult.

In summary, the findings described here further our understanding of the intricate structure of this essential multisubunit
complex in higher organisms and strengthen evidence in support of the application of the current structural model of H\(^+\)-ATPase derived from yeast regarding the inclusion of the N-terminal of the α subunit in the peripheral stalk, which links the V\(_1\) and V\(_0\) sectors. They also reinforce the hypothesis that specialized pumps containing different combinations of tissue-restricted subunits carry out different but important functions within an organism and highlight differences in the constitution of H\(^+\)-ATPases from specialized cell types between human and rodent.

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