The a-Subunit of the V-type H\(^+\)-ATPase Interacts with Phosphofructokinase-1 in Humans*

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V-type or H\(^+\)-ATPases are a family of ATP-dependent proton pumps that move protons across the plasma membrane at specialized sites such as kidney epithelial cells and osteoclasts as well as acidifying intracellular compartments. The 100-kDa polytopic a-subunit of this group of ATPases is suggested to play an important role in coupling the two functions of the pump, ATP hydrolysis and proton transport. In man, different a-subunit isoforms are encoded by four genes. ATP6V0A4 encodes a4, which is expressed apically in renal intercalated cells and osteoclasts as well as acidifying intracellular compartments. The 100-kDa polytopic a-subunit of this isoform is mainly responsible for ATP hydrolysis. The V\(_0\) domain contains up to five additional subunits (a, c, c\(_1\), c\(_2\), and d) and is primarily responsible for proton translocation across the membrane in which it is anchored (2).

The yeast V-ATPase is the best-characterized proton pump in eukaryotes. A more detailed structural model, mainly derived from studies of both yeast and bovine orthologs, is that H\(^+\)-ATPases can better be defined by several regions. They are, as before, a catalytic core composed of A and B subunits; a central rotor or stalk, suggested to be composed of D and F subunits; a peripheral “stator” likely to be composed of the N terminus of the a-subunit together with C, E, G, and H, and a proton-translocating domain composed of the C terminus of a, with c, c\(_1\), and c\(_2\) subunits (3-5). However, to date, mammalian orthologs of yeast c\(_1\) have not been identified.

H\(^-\)-ATPases are structurally and evolutionarily related to the F\(_\mathrm{F}_1\)-ATP synthase, responsible for ATP synthesis in mitochondria, chloroplasts, and bacteria (6–9). Interestingly, although the F\(_\mathrm{F}_1\)/F\(_\mathrm{F}_0\)-ATP synthase retains the capacity either to synthesize ATP (using the energy provided by passive H\(^+\) movement) or to hydrolyze it, providing energy for proton pumping in the opposite direction, H\(^+\)-ATPases can only perform the latter function. Whether the energy source for H\(^-\)-ATPase function is derived from mitochondrial or glycolytic ATP is unclear, and the regulatory mechanisms involved are not well understood.

Parallels for the mechanism by which H\(^-\)-ATPases carry out ATP-dependent proton transport can be drawn from the mechanism proposed for F\(_\mathrm{F}_1\)/F\(_\mathrm{F}_0\)-ATP synthase hydrolytic function (8, 10). In the latter, energy produced from ATP hydrolysis by the catalytic core is thought to drive the rotation of the central stalk. This results in the rotation of the proton channel, which lies adjacent to the a-subunit. The a-subunit is thought to bring the protons to this channel, which releases them on the opposite side of the membrane as it rotates (for review, see Ref. 11). Thus, it can be seen that the a-subunit is crucial for proton translocation. Although there is no obvious sequence or structural homology between the a-subunits of H\(^+\) and F\(_\mathrm{F}_1\)/F\(_\mathrm{F}_0\)-ATP synthases, functional studies have suggested that they do in fact behave analogously (2, 12, 13).

Various a-subunit paralogs have been found in different species (14–17). In both mouse and man, different a-subunit isoforms are encoded by four genes (18, 19). Although the a1 isoform is ubiquitously expressed, a separate gene (ATP6V0A4 in humans) encodes a4, the paralog that is expressed predominantly apically by intercalated cells in both human and mouse kidney. Defects in the genes encoding a4 and the osteoclast-enriched a3 isoform (TCIRG1) are associated with the recessively inherited human diseases distal renal tubular acidosis and infantile malignant osteopetrosis, respectively (18, 20, 21), underscoring the functional importance of this subunit in kidney and bone at least.

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The H\textsuperscript+-ATPase a-subunit is an ~100-kDa polytopic membrane protein that has a bipartite structure containing an N-terminal hydrophilic domain and a C-terminal hydrophobic domain with 6–9 putative transmembrane helices (14). It has not been definitively determined whether the N and C termini lie on the same or opposite sides of the membrane. As noted from the yeast ortholog Vph1p, the N-terminal domain is likely to contribute to the formation of the stator, providing a structural support for the H\textsuperscript+-ATPase (3). Site-directed and random mutagenesis studies of Vph1 showed that mutations in the last two transmembrane regions inhibit both proton transport and ATPase activity (13). Also, a cluster of five mutations was identified between residues 800 and 814 in the C-terminal soluble segment, which affected either assembly or stability of the H\textsuperscript+-ATPase complex. Two of these mutations may also affect targeting of the a-subunit (22). In addition, Manolson et al. (14) report that disruption of the a-subunit in yeast affects assembly of V\textsubscript{1} onto the vacuolar membrane. Taken together, all these results suggest that in yeast at least, the C-terminal soluble tail of the a-subunit plays a crucial role in function and organization of the ATPase complex.

Information concerning potential functional or regulatory contributions of the C-terminal domain of the different tissue-specific isoforms of the mammalian H\textsuperscript+-ATPase a-subunit is scarce. We have been particularly interested in the a4 isoform because of its essential contribution to renal acid-base homeostasis. Noting first, the suggested importance of the C terminus to the H\textsuperscript+-ATPase as outlined above, and second, that this region is the most homologous among the four a-subunit isoforms, we therefore sought further to explore the role of this domain of human a4 (here designated a4(C))\textsuperscript{1}. We initially used random peptide phage display analysis to identify potential binding partners, followed by both \textit{in vitro} binding and \textit{ex vivo} assays. We report here the identification of a new binding partner, phosphofructokinase 1 (PFK-1), which we also demonstrate interacts with the a4 subunit. The association between these two proteins indicates a direct link between the H\textsuperscript+-ATPase and glycolytic pathway via the a-subunit that can furnish the ATP necessary for pump function.

**EXPERIMENTAL PROCEDURES**

\textit{a4(C) Construction, Protein Expression, and Purification—}cDNA encoding the last 45 amino acids (796–840) of human a4 was amplified by high fidelity PCR using primers (5'-3') CGGGATCCATGGAGGGCGCACTG-3' and CGGAATTCCTCGACTGGGCGGCGTGTCATCG to include the 5' EcoRI and 3' EcoRI restriction sites. The insert was used to clone the gel-purified PCR product in-frame into the GST expression vector pGEX-4T-1 (Amersham Biosciences). Clones containing the fusion construct were identified by both colony PCR and restriction enzyme digestion and verified using ABI Prism\textsuperscript{TM} BigDye\textsuperscript{TM} Terminator cycle sequencing (Applied Biosystems).

Large scale fermentation was used to express the fusion protein; after an 18-h initial incubation at 37 \textdegree C, cultures were diluted 1 in 50 and grown in a fermenter (FT Applikon) for 2.5 h at 37 \textdegree C to an A\textsubscript{600} of 0.8. The incubation temperature was lowered to 30 \textdegree C for 30 min; isopropyl-1-thio-\beta-D-galactopyranoside was added (final concentration 0.1 mM), and incubation was continued for another 2 h. Bacterial cells were pelleted by centrifugation at 2250 \times g for 10 min and re-suspended in a buffer containing PBS, 1 mM EDTA, 0.1% \beta-mercaptoethanol, and Complete EDTA-free protease inhibitor mixture tablets (Roche Diagnostics). Cell lysis was achieved by sonication (Misonix XL2020) for 21 \times 10 s. After centrifugation at 44,000 \times g for 30 min, the supernatant was collected. To purify the GST fusion protein, the lysate was first passed through a GSTTrap pre-packed Column (Amersham Biosciences) according to the manufacturer’s protocol. The eluate was concentrated using VivaSpin 20 columns (VivaScience) and then subjected to thrombin digestion.

Thrombin activity was terminated by twice adding phenylmethylsulfonyl fluoride (0.2 mM) at 4 \textdegree C for 15 min. After brief centrifugation to remove aggregates, the supernatant was loaded onto a C18 HPLC column (Phenomenex, PRODigy 5-μm ODS3, 21.2 \times 250 mm, 100 Å). Protein was eluted across a gradient of 20–60% CH\textsubscript{3}CN in 0.1% trifluoroacetic acid. The recombinant a4(C) fragment was analyzed for purity and molecular weight by liquid chromatography-mass spectrometry (HP1100 coupled with LCQ, Finnigan MAT), lyophilized, and stored at -20 \textdegree C. 10 μg each of GST-a4(C) fusion protein eluate, thrombin digest, and the a4(C) protein from HPLC purification were analyzed by Western blotting of a 16% SDS-polyacrylamide gel according to standard methods. The blot was probed with the polyclonal antibody RA2922, directed against the last 14 amino acid residues of human a4 as previously described (18), and then followed by HRP-conjugated secondary antibody (Dako). The blot was developed using enhanced chemiluminescence (Kirkgaard & Perry Laboratories).

\textit{a4(C) Synthesis—}A 46-amino acid peptide corresponding to the published sequence of the human ATP6V0A1 C terminus (a11), GenBank\textsuperscript{TM} Q93050 (www.ncbi.nlm.nih.gov/entrez) containing an additional N-terminal acetylated cysteine residue was synthesized and H\textsuperscript{35}S-labeled by Covallab UK Ltd. (Cambridge, UK). Phage Display Screening—Lyophilized purified a4(C) was reconstituted in water and diluted in 0.1 mM NaHCO\textsubscript{3} (pH 8.6) to a final concentration of 0.1 mg/ml. Thereafter, 10 μg were coated onto a micro-plate well at 4 \textdegree C overnight in a humidifier. Unbound protein was removed, and the well was blocked with blocking buffer for 2 h at 4 \textdegree C with gentle rocking. The Ph.D.-7\textsuperscript{TM} phage display peptide library (New England Biolabs) was used according to the manufacturer’s protocol. About 2 \times 10\textsuperscript{11} phage virions were added into the blocked well for the first round of panning and incubated for 1 h at room temperature with gentle agitation. Bound phages were eluted using glycine buffer (0.2 mM glycine-HCl (pH 2.2) containing 1 mM MgCl\textsubscript{2}) and passed through a GSTrap pre-packed Column (Amersham Biosciences) according to the manufacturer’s option. Bound phages were amplified using 200 μl of 2.2'-azino-bis-(3-ethylthio-azonie-6-sulfonic acid) 0.022% (w/v) in 50 mM sodium citrate (pH 4.0) containing 0.05% H\textsubscript{2}O\textsubscript{2}. 10\textsuperscript{11} amplified phage virions were used for a 2nd and 3rd round of panning. After the 3rd panning, individual phage clones were isolated and characterized by ABI Prism\textsuperscript{TM} Terminator cycle sequencing. Protein BLAST (www.ncbi.nlm.nih.gov/BLAST) was used to search for homology (using the “short nearly exactly matches” option) between identified amino acid sequences and protein data base sequences.

Phage Enzyme-linked Immunosorbent Assay—Enzyme-linked immunosorbent assay experiments were carried out essentially under the same conditions as those used in panning procedures of Phage Display, employing 2-fold dilutions of the consensus sequence displaying phage. Nonspecific binding was evaluated in parallel wells lacking a4(C). For detection, HRP-conjugated anti-M13 antibody (Amersham Biosciences) 15,000 in PBS 1:15,000 was added to each well. Bound M13 phages were detected using 20,000 amplified M13 phages/ml and washed with PBS-0.05% Tween 20 containing 2% dried skimmed milk was added into each well and incubated for 2 h at room temperature with gentle agitation. After 6 washes in PBS, bound phages were visualized using 200 μl of 2.2'-azino-bis-(3-ethylthio-azonie-6-sulfonic acid) 0.022% (w/v) in 50 mM sodium citrate (pH 4.0) containing 0.05% H\textsubscript{2}O\textsubscript{2}. 400 values were measured after 20 min of incubation using a microplate reader (Anthos HTII).

PFK-1 Immunoreactivity in Human Kidney—A goat polyclonal antibody directed against rabbit muscle-type PFK-1 (Chemicon International Ltd.) was tested for immunoreactivity against human kidney cytosolic, and membrane protein fractions, which were prepared as previously described (19). 10 μg of each sample were subjected to 12% SDS-PAGE and PFK-1 antibody at 1:2000 dilution. Solubilization of Human Kidney Membrane Proteins and Immunoprecipitation—60 μg of the goat anti-rabbit-muscle-type PFK-1 were immobilized onto agarose beads using the Seize Primary Mammalian immunoprecipitation kit (Pierce) according to the manufacturer’s guidelines.

Based on earlier work (23), 400 μg of human kidney membrane protein prepared as previously described (19) were solubilized in a solubilization buffer containing 10 mM Tris-CI (pH 7.4), 1 mM EDTA, 1 mM diethiothreitol, 10% glycerol, either 1% n-nonyl-β-D-glucopyranoside or 0.6% CHAPS, and protease inhibitor mixture tablet. All steps were carried out at 4 \textdegree C. After gentle rotation for 30 min and centrifugation a 10,000 × g for 1 h, 15 μl of unconjugated beads from the same kit were added to the recovered supernatants and incubated for 1 h. These beads were replaced by 50 μl of the PFK-1 antibody-coupled beads before overnight incubation. The beads were then washed 3 times with 1 ml of buffer containing 20 mM Tris-CI, pH 7.4, 5 mM Na\textsubscript{2}PO\textsubscript{4}, and

\textsuperscript{1} The abbreviations used are: a4(C), 45 C-terminal amino acids of the H\textsuperscript+-ATPase a-subunit isoform; a11, 45 kDa C-terminal amino acids of the H\textsuperscript+-ATPase a1 subunit isoform; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; PFK, phosphofructokinase; TRITC, tetramethylrhodamine isothiocyanate.
either 0.3% n-octyl-4-D-glucopyranoside or 0.3% CHAPS, 3 times with the same buffers containing 500 mM NaCl, and finally, 3 times with buffers without NaCl. Bound proteins were eluted from the agarose beads by boiling in SDS sample buffer (0.175 M Tris-HCl (pH 6.8), 5.14% (w/v) SDS, 18% (v/v) glycerol, 0.3 M dithiothreitol, 0.006% (w/v) bromphenol blue) at 95 °C, and supernatants were subjected to SDS-PAGE. Western blotting was performed with rabbit RA2922 antibody or anti-PFK-1 antibody as above.

Biotinylation of a4(C) or a1(C) and in Vitro Binding of PFK-1—Biotin cadaverine (Molecular Probes) was dissolved in 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (Sigma) to a final concentration of 2.4 mg/ml. Purified a4(C) was dissolved at 0.5 mg/ml in H2O. Biotin cadaverine/EDAC solution was added to 25 μg a4(C) solution and incubated at room temperature for 1 h. Excess biotin activity was quenched by the addition of 10 μl of 1 M sodium acetate (pH 5.0) followed by incubation at room temperature for 2 h. Water replaced a4(C) as a control. This protocol was repeated with HPLC-purified synthetic a1(C) peptide. Labeled samples were stored at −20 °C before binding assays.

Rabbit muscle-type PFK-1 bound to agarose beads (Sigma) was equilibrated with ice-cold PBS and collected by centrifugation at 2000 × g for 2 min. Analysis of protein-protein interactions was achieved by incubation of 25 μg of biotin-labeled a4(C) or a1(C) with 50 μl of equilibrated PFK-1-bound agarose beads for 1 h at 4 °C with gentle rotation. The beads were collected by centrifugation and washed (4 × 1 ml) with ice-cold PBST. Bound proteins were eluted from the agarose beads by boiling the beads in SDS sample buffer for 10 min at 95 °C, and supernatants were spotted onto nitrocellulose membranes. 1:1000 HRP-conjugated avidin (Sigma) was used to probe the membrane according to standard methods.

Immunohistochemistry—Samples of normal human kidney were obtained from nephrectomy specimens resected because of renal tumors. Informed written consent was obtained with the approval of the Addenbrooke's hospital histopathology department Tissue Bank Committee. Cortico-medullary sections 1 mm thick were embedded in Tissue-tek OCT compound and snap-frozen in liquid nitrogen-cooled isopentane. 5-μm sections were cryostat-cut and thaw-mounted on aminopropyltriethoxysilane-coated glass slides. Sections were stored at −80 °C until use.

Sections were fixed in 100% methanol at −20 °C for 5 min and rehydrated by a 5-min immersion in TBST (0.1 M Tris-buffered saline plus 0.001% Tween 20 (pH 7.5). All subsequent incubations and rinses were in TBST. Nonspecific antibody binding was blocked by ambient temperature incubation in blocking buffer containing 10% fetal calf serum in TBST for 15 min. Goat anti-rabbit muscle PFK-1 antibody and rabbit RA2922 antibody were applied at 1:100–1:5000 dilutions in blocking buffer overnight at 4 °C. TRITC-labeled anti-rabbit or fluorescein isothiocyanate-labeled anti-goat secondary antibodies (Vector Laboratories) were applied at 1:100 dilution for 40 min at ambient temperature. After mounting in Citifluor mounting medium, bound antibodies were visualized using a Leica TCS-NT confocal laser scanning microscope. Replacement of primary antibody with the appropriate pre-immune serum provided negative controls.

RESULTS

Expression and HPLC Purification of a4(C)-GST Fusion Protein—To express a4(C) in bacteria, the coding sequence for this region was ligated in-frame to the coding sequence of GST in the GST-4T-1 expression vector. The GST-a4(C) fusion protein was expressed in this system only at very low levels. Therefore, to obtain sufficient a4(C) protein, a large scale fermentation technique was employed. Western blot analysis using antibody RA2922 (raised against the C-terminal 14 residues of human a4) demonstrated the presence of the fusion protein (Fig. 1A, lanes 1 and 2, before and after thrombin digestion) in both intact and degraded forms. A band corresponding to a4(C) is evident in the post-thrombin-digested sample (lane 2). This thrombin-digested product was subjected to HPLC purification. Fig. 1B demonstrates that of the three main peaks observed, the second peak (arrow) corresponded to the recombinant a4(C). The first peak represented a fraction of smaller mass than expected but was recognized by RA2922 (data not shown), indicating the presence of an alternative thrombin cleavage site within a4(C). The third peak corresponded to non-digested a4(C). The first peak corresponded to the fraction containing intact a4(C) protein. This fraction was further analyzed by mass spectrophotometry. Its molecular mass, 5.41 kDa, confirms it as a4(C).

GST-a4(C) fusion protein. As can be seen in Fig. 1C, further passage of the second fraction through an HPLC column linked to a mass spectrophotometer confirmed a single peak representing the a4(C) fragment, with a mass of 5.41 kDa. This corresponded well to the 5.264 kDa predicted by the program Compute pI/Mw tool (us.expasy.org/tools/pi_tool.html), the difference accounted for by two additional amino acids, Gly and...
Phage Display Analysis of α4(C)—A 7-mer random peptide M13 phage display library was used to screen immobilized α4(C) for potential interaction partners. For this purpose recombinant α4(C) protein was fixed to microtiter plates through hydrophobic interactions and subjected to three rounds of “panning” (recovery of bound phage followed by their reapplication for the next round). Sequence analysis of enriched phage clones after the third panning yielded the peptide sequence SWLELRP, which was found in 7 of 17 clones sequenced (Table I). Using the “short nearly exact matches” option, comparative BLAST analysis revealed an almost complete match to the consensus phage 7-mer peptide SWLELRP, which is a key participant in the glycolytic pathway. Aligning this sequence with other selected phage clone sequences revealed a longer consensus motif EXWWLKLKP (Table I). This matched region within PFK-1 is highly conserved among mammalian PFK-1 orthologs and paralogs (Table II).

In Vitro Confirmation of α4(C)-Peptide Interaction—The interaction of α4(C) with the consensus phage 7-mer peptide representing PFK-1 was confirmed by an enzyme-linked immunosorbent binding assay (Fig. 2). This showed a significant binding affinity of this peptide to immobilized α4(C) protein. Maximum binding was observed in the range of $1 \times 10^{11}$ phage virions/well, which is similar to the concentration of phage virions used in each of the panning procedures in the phage display procedure.

PFK-1 Co-immunoprecipitates with α4 in Human Kidney—Before examining the ex vivo interaction between PFK-1 and the α4 subunit in human kidney, we confirmed that a goat polyclonal antiserum directed against rabbit muscle-type PFK-1 could recognize human PFK-1 in human kidney cytosolic and membrane protein fractions (data not shown).

To examine whether α4 and PFK-1 interact in vivo, we next carried out an immunoprecipitation assay using this α-PFK-1 antibody for precipitation and RA2922 for detection. The resulting blot, showing a single major band at $\sim 116$ kDa, recognized by the α-α4 antiserum (Fig. 3A), conforms to previous analysis using this antibody (19). An identical blot probed with the α-PFK-1 antiserum revealed a band of the correct size, confirming the presence of the enzyme (Fig. 3B). This demonstration of the co-precipitation of these two proteins indicates their interaction. Interestingly, α4 was present only in the protein sample prepared with n-nonyl-β-D-glucopyranoside as the detergent in both solubilization and washing buffers and not when CHAPS was used (data not shown). Specificity of the assay was confirmed by absence of α4 when the precipitating antibody was omitted (lanes).

PFK-1 Pull-down Assay—We next asked whether the interaction between α4(C) and PFK-1 was also true of the C terminus of the ubiquitously expressed α1 subunit (α1(C)). We wished to perform similar immunoprecipitation studies but found that the available α1 antibody (a kind gift of M. Futai) appeared to cross-react with α4 (data not shown). We therefore designed a PFK-1 pull-down assay. HPLC-purified α4(C) or α1(C) were first labeled with biotin, and after confirmation of successful biotinylation (Fig. 4, panel A), were then incubated with agarose beads to which PFK-1 was bound. After extensive washing, bound proteins were eluted by boiling, and the supernatant was spotted on nitrocellulose membrane and analyzed using avidin-α-biotin. Binding of PFK-1 to both α4(C) and α1(C) was evident from this assay (panels C and E). As can be seen
from panels D and F, this binding was specific, since when an unrelated protein (protein A) conjugated to identical agarose beads was used, no significant binding of a4(C) or a1(C) was observed. Finally, panel G confirmed there was no binding between PFK-1 and the avidin-HRP conjugate alone.

**Immunolocalization of a4 and PFK-1 in Human Kidney**

The distributions of a4 and PFK-1 in human kidney were compared by double-label immunohistochemistry using the antibodies to human a4 (RA2922) and to rabbit muscle type PFK-1 employed earlier. As previously documented (18), specific antibodies to human a4 (RA2922) and to rabbit muscle type PFK-1 were employed. As previously documented (18), specific antibodies to human a4 (RA2922) and to rabbit muscle type PFK-1 were employed. As previously documented (18), specific antibodies to human a4 (RA2922) and to rabbit muscle type PFK-1 were employed.

At left, the typical appearance of a4 is shown, localized to the apical surfaces of intercalated cells in the collecting duct, as previously observed. As expected, PFK distribution was widespread throughout all nephron segments, with some enrichment apically in the collecting duct (center panel). The merged image (right panel) demonstrates co-localization of a4 and PFK-1 (arrows) in a-intercalated cell. Scale bar = 10 μm.

**DISCUSSION**

The a-subunit of the proton pump is deemed crucial for coupling of ATP hydrolysis (V1) and proton transport (V0). Because its soluble C-terminal tail has been shown to play potential roles in the assembly, stabilizing, or targeting of the a-subunit as well as proton translocation (22), we chose to investigate possible binding partners for this part of the molecule. Physical interactions between H\(^+\)-ATPase subunits or between subunits and other proteins have been variously reported (3, 24–28). Intermolecular interactions among different subunits are more likely to provide a structural support for the proton pump, whereas interactions with other proteins might provide insights into H\(^+\)-ATPase assembly, transport, targeting, or regulation.

This study presents multiple lines of evidence that identify the glycolytic enzyme PFK-1 as a novel binding partner for the C terminus of the pump a-subunit. We initially focused on the a4 subunit because in contrast to its intracellular counterpart a1, it has a differently targeted distribution to the apical surface of polarized cells in the kidney. We confirmed the initial phage peptide interaction by proceeding to *in vitro* binding studies of a4(C) with intact PFK-1 and also demonstrated that a4 and PFK-1 can be co-precipitated from human kidney membranes. Last, immunolocalization in human kidney suggests some enrichment of PFK-1 at the same sites as high intensity a4 immunoreactivity in acid-secreting cells of the distal nephron.

We subsequently showed that PFK-1 also binds to the ubiquitous a1-subunit C terminus. However, the available polyclonal anti-a1 antibody (29) appeared to recognize both the a1- and a4-subunits, and we were unable to make a meaningful interpretation of immunoblots. Instead we used a specific *in vitro* assay to demonstrate the interaction. Of the 45 amino acids in the a-subunit C terminus, the first 23 are identical in the a1 and a4 isoforms, with only 59% identity thereafter, and it is therefore likely that this region contains the binding domain.

Our results strongly suggest that there is a direct link between proton transport and glycolysis and implies that the energy source for pump function is glycolytic rather than mitochondrial. Lu *et al.* (24) report an interaction at the protein level between aldolase and the ubiquitously expressed E subunit of the V-ATPase, again supporting a direct coupling between the proton pump and glycolysis. Aldolase is the next enzyme in the glycolytic pathway that, after the action of PFK-1, cleaves fructose 1,6-bisphosphate. Taken together with our results, it is probable that the many pump and glycolytic components form a “metabolon” to maximize the efficiency of energy provision. Whether other enzymes involved, such as hexokinase, also physically interact with H\(^+\)-ATPase components, is unknown.

The involvement of glycolytic enzymes with the proton pump is of especial interest in the intercalated cell, which has been labeled the “mitochondrion-rich” cell type in the nephron (30). Other energy-dependent transport functions of this cell are likely to depend on the mitochondrial supply of ATP, but in the case of the apical H\(^+\)-ATPase that is responsible for urinary acidification, we propose the alternative.

Several other lines of evidence support this hypothesis. First, functional assessment of proton transport in the isolated turtle urinary bladder showed that it could be driven by the energy from both aerobic and anaerobic glycolysis (31, 32). Second, an investigation of the effect of glucose on the reversible assembly of the V1 and V0 domains of the pump complex in yeast (33) suggested coupling between H\(^+\)-ATPase activity and glycolysis. Interestingly, accumulation of glucose 6-phosphate was insufficient to maintain or induce this assembly, suggesting that further glucose metabolism is required. In addition, the signaling involved in V-ATPase assembly did not appear to involve the Ras-cyclic AMP pathway, Snf1p, protein kinase C, or Rts1p, a stress response protein. This study suggested that the transient cytosolic pH drop resulting from the initiation of glycolysis could provide a signal for activation of V-ATPase by triggering the glucose-induced assembly of the V1 domain and V0 domains. Thus, we can speculate that the activity of PFK-1 may have a role as an indirect regulator of the proton pump.

In the glycolytic pathway, PFK-1 catalyzes the phosphorylation of fructose 6-phosphate by Mg-ATP to form fructose 1,6-bisphosphate and Mg-ADP. This reaction is the rate-limiting
step in glycolysis, which is therefore critically dependent on the level of activity of PFK-1. This in turn is allosterically controlled both by the ratio of ATP to AMP and by several other metabolites including citrate and fructose 2,6-bisphosphate (for review, see Ref. 34). Mammalian PFKs have yet to be crystallized, but comparison with the bacterial form suggests that the binding region we have identified for a4(C) is distant from the described active sites of substrate binding and allosteric regulation, which involve residues Arg-48, Arg-97, Arg-433, Arg-481, and Ser-541 among others (35–38). This implies that the binding of a4(C) to PFK-1 should not directly affect its kinase function but may instead anchor the enzyme to the pump. Future studies will be required to address this issue.

The enzymatically active form of PFK-1 is a tetramer or high order oligomer with a subunit molecular mass of 85 kDa (39). In humans, isozymes of PFK-1 have been grouped as muscle type (M-PFK-1), liver type (L-PFK-1), and platelet type (P-PFK-1) (40). All three types have been detected in human kidney (34, 41), although it is not known which is the most prevalent. Rabbit M-PFK-1, to which the only commercially available antibody was raised, shares a sequence identity of about 96% with human M-PFK-1. The sequence identity between rabbit M-PFK-1 and human L-PFK-1 is not as high, about 69%, but comparative structural analysis has revealed a high degree of similarity, from which it is implied that this will also be true at the functional level (42). However, the potential for differences between the PFK isoforms means that the relative power of the available antibody to interact with the H+-ATPase-PFK complex may be less than optimal.

The α-subunit was initially described as the “large accessory” subunit of the H+-ATPase, and its presence in the kidney was once disputed (23, 43). In recent years it has become evident from the study of human diseases that its presence is essential for normal pump function at the cell surface of renal intercalated cells and osteoclasts at least (18, 20, 21). Through the use of different solutions in our immunoprecipitation experiments, we have shown that the detection of this subunit is critically dependent on the detergent employed in disrupting the cell. In particular, unsuccessful detection after α4 co-immunoprecipitation with PFK-1 when using buffers containing CHAPS suggests that this interaction, at least, is sensitive to this detergent. In a similar way, the differences in subunit composition reported for H+-ATPases in the kidney may in fact have related to methodological differences in tissue preparation.

Finally, topical studies of Vph1p employing cysteine mutagenesis and chemical labeling have led to a model for the α-subunit in the yeast vacuole that contains nine transmembrane helices. In this model, the N-terminal domain lies on the cytoplasmic side of the membrane, and the C terminus lies on the luminal side of the vacuole (44). However, data suggesting six transmembrane domains, with cytoplasmic orientation of both N and C termini, have also been reported in yeast and Dictyostelium (45, 46). In the absence of crystallographic or other structural information, it has not been clear whether in the case of α4 the C-terminal tail would be found below the apical cell membrane in intercalated cells or protruding into the urinary space. Our finding of the interaction between PFK-1 and α4(C) provides new evidence for an intracellular location of α4(C).

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REFERENCES

1. Stevens, T. H., and Forgac, M. (1997) Annu. Rev. Cell Dev. Biol. 13, 779–808
2. Zhang, J., Feng, Y., and Forgac, M. (1994) J. Biol. Chem. 269, 23518–23523
3. Loholt-Marticorena, C., Wagner, K. M., Correa, L., Chen, W., and Manolson, M. F. (2000) J. Biol. Chem. 275, 15449–15457
4. Nishi, T., and Forgac, M. (2002) Nat. Rev. Mol. Cell Biol. 3, 94–103
5. Lu, M., Verpari, S., Zhang, L., Lefranc, S. L., Aris, J., and Gluck, S. L. (2002) J. Biol. Chem. 277, 38469–38415
6. Weber, J., and Senior, A. E. (1997) FEBS Lett. 412, 169–172
7. Fillingame, R. H. (1997) J. Exp. Biol. 200, 217–224
8. Cross, R. L., and Duncan, T. M. (1996) J. Bioenerg. Biomembr. 28, 403–408
9. Putai, M., and Omote, H. (1996) J. Bioenerg. Biomembr. 28, 409–414
10. Vik, S. B., and Antonio, B. J. (1994) J. Biol. Chem. 269, 30364–30369
11. Oka, T., Toyomura, T., Honjo, K., Wada, Y., and Futai, M. (2001) J. Biol. Chem. 276, 33070–33085
12. Bowman, E. J., Siebers, A., and Altendorf, K. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7972–7976
13. Leng, X. H., Manolson, M. F., Liu, Q., and Forgac, M. (1996) J. Biol. Chem. 271, 22487–22493
14. Manolson, M. F., Proteau, D., Preston, R. A., Stentis, A., Roberts, B. T., Hoyt, M. A., Preaus, D., Mulholland, J., Botstein, D., and Jones, E. W. (1992) J. Biol. Chem. 267, 14298–14303
15. Manolson, M. F., Wu, B., Proteau, D., Taillon, B. E., Roberts, B. T., Hoyt, M. A., and Jones, E. W. (1994) J. Biol. Chem. 269, 14406–14407
16. Mattsson, J. P., Li, X., Peng, S. B., Nilsson, F., Andersen, P., Lundbrandt, L. G., Stone, D. K., and Keeling, D. J. (2000) Eur. J. Biochem. 267, 4115–4126
17. Oka, T., Toyomura, T., Honjo, K., Wada, Y., and Futai, M. (2001) J. Biol. Chem. 276, 29307–29315
18. Smith, A. N., Skag, J., Chote, K. A., Nayar, A., Bakkaloga, L., Oxen, S., Hulton, S. A., Sanjad, S. A., Al-Sabban, E. A., Lifton, R. P., Scherer, S. W., and Karet, F. E. (2000) Nat. Genet. 26, 71–75
19. Smith, A. N., Finberg, K. E., Wagner, C. A., Lifton, R. P., Dequidan, M. A., Su, Y., and Karet, F. E. (2001) J. Biol. Chem. 276, 42382–42388
20. Prattini, A., Orchard, P. J., Sobacchi, C., Galili, S., Abnun, M., Mattsson, J. P., Keeling, D. J., Ziche, L., Notarangelo, L. D., Vezzoni, P., and Villa, A. (2000) Nat. Genet. 23, 343–346
21. Kernak, U., Schulz, A., Friedrich, W., Uhlaas, S., Kremsen, B., Voit, T., Hassan, C., Bode, U., Jentsch, T. S., and Kubisch, C. (2000) Hum. Mol. Genet. 9, 2059–2066
22. Leng, X. H., Manolson, M. F., and Forgac, M. (1998) J. Biol. Chem. 273, 6717–6723
23. Glick, S., and Caldwell, J. (1987) J. Biol. Chem. 262, 15780–15789
24. Lu, M., Holiday, L. S., Zhang, L., Dunn, W. A., Jr., and Gluck, S. L. (2001) J. Biol. Chem. 276, 30407–30413
25. Breton, S., Wiederhold, T., Marshansky, V., Neumu, N. N., Ramesh, V., and Brown, D. (2000) J. Biol. Chem. 275, 18219–18224
26. Holiday, L. S., Lu, M., Lee, B. S., Nelsen, R. D., Solivan, S., Zhang, L., and Gluck, S. L. (2000) J. Biol. Chem. 275, 32331–32337
27. Lee, B. S., Gluck, S. L., and Holiday, L. S. (1999) J. Biol. Chem. 274, 29164–29171
28. Xu, T., Yasuiyeva, E., and Forgac, M. (1999) J. Biol. Chem. 274, 28999–29015
29. Tanaka, T., Oka, T., Yamaguchi, C., Wada, Y., and Putai, M. (2000) J. Biol. Chem. 275, 8760–8765
30. Brown, D., and Breton, S. (1996) J. Exp. Biol. 199, 2345–2358
31. Beaugues, R., and Al-Awqati, Q. (1996) J. Gen. Physiol. 88, 421–439
32. Steinmetz, P. R., Husted, R. F., Mullett, P. D., and Beaugues, R. (1981) J. Membr. Biol. 59, 27–34
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