Physical and Functional Links between Anion Exchanger-1 and Sodium Pump

Ya Su,* Rafia S. Al-Lamki,† Katherine G. Blake-Palmer,* Alison Best,* Zoe J. Golder,* Aiwu Zhou,‡ and Fiona E. Karet Frankl*†

Departments of *Medical Genetics and †Haematology, and ‡Division of Renal Medicine, University of Cambridge, Cambridge, United Kingdom

ABSTRACT

Anion exchanger-1 (AE1) mediates chloride-bicarbonate exchange across the plasma membranes of erythrocytes and, via a slightly shorter transcript, kidney epithelial cells. On an omnivorous human diet, kidney AE1 is mainly active basolaterally in α-intercalated cells of the collecting duct, where it is functionally coupled with apical proton pumps to maintain normal acid-base homeostasis. The C-terminal tail of AE1 has an important role in its polarized membrane residency. We have identified the β1 subunit of Na+, K+-ATPase (sodium pump) as a binding partner for AE1 in the human kidney. Kidney AE1 and β1 colocalized in renal α-intercalated cells and coimmunoprecipitated (together with the catalytic α1 subunit of the sodium pump) from human kidney membrane fractions. ELISA and fluorescence titration assays confirmed that AE1 and β1 interact directly, with a \( K_d \) value of 0.81 \( \mu \)M. GST-AE1 pull-down assays using human kidney membrane proteins showed that the last 11 residues of AE1 are important for β1 binding. siRNA-induced knockdown of β1 in cell culture resulted in a significant reduction in kidney AE1 levels at the cell membrane, whereas overexpression of kidney AE1 increased cell surface sodium pump levels. Notably, membrane staining of β1 was reduced throughout collecting ducts of AE1-null mouse kidney, where increased fractional excretion of sodium has been reported. These data suggest a requirement of β1 for proper kidney AE1 membrane residency, and that activities of AE1 and the sodium pump are coregulated in kidney.

Anion exchanger-1 (AE1) is a sodium-independent member of a family of 1:1 chloride-bicarbonate exchangers. In mammals, AE1 is expressed at high levels in erythrocytes (eAE1) and kidney (kAE1). Both AE1 isoforms are encoded by SLC4A1 but are under the control of separate promoters, resulting in human kAE1 lacking the first 65 residues that are present in eAE1.1 Mutations in SLC4A1 affecting eAE1 and kAE1 are associated with autosomal dominant hereditary spherocytosis and distal renal tubular acidosis (dRTA), respectively.2 However, in most cases, single mutations resulting in dRTA do not also produce hereditary spherocytosis, and vice versa. In the kidney, kAE1 is mainly found at the basolateral membrane of the type A acid secreting intercalated cells (α-ICs) of the collecting duct in the distal nephron, where it is functionally coupled to the apical proton pumps to maintain normal bodily acid-base homeostasis. Failure of α-IC function results in dRTA, a condition characterized by impaired urinary acid secretion, hyperchloremic metabolic acidosis, hypokalemia, growth retardation, nephrocalcinosis, and nephrolithiasis.3 Mutant kAE1 proteins associated with autosomal dominant dRTA usually exhibit normal or only modestly reduced Cl−/HCO3− transport activity, but show severe trafficking defects. To date, at least

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Correspondence: Dr. Fiona E. Karet Frankl, Cambridge Institute for Medical Research, Cambridge Biomedical Campus, Hills Road, Cambridge, CB2 0XY, UK. Email: fek1000@cam.ac.uk

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two mistargeting phenotypes, intracellular retention and aberrant membrane accumulation, have been observed and are the major pathogenic mechanisms.5–6

AE1 is composed of a large cytosolic N-terminal domain, a central transmembrane region that is predicted to span the lipid bilayer 12–14 times and is responsible for the anion exchange, and a short cytosolic C-terminal tail (AE1C). Multiple binding sites for other proteins including cytoskeletal proteins ankyrin-1 (ANK1), glycolytic enzymes, and hemoglobin have been identified in the N-terminal domain of eAE1.7–11 However, none of these appears to interact with the N terminus of kAE1.9,12,13 The crystal structure of the N-terminal domain of eAE1 has been solved. The monomer structure consists of 11 β-strands and 10 helical segments.14 In the case of kAE1, truncation of the first 65 residues results in deletion of the middle β-strand in the central β-barrel, which may result in a significant change in the protein’s function. In addition, it is notable that AE1 mutations associated with autosomal dominant dRTA are spread over both transmembrane and C-terminal domains, but no such mutations have thus far been identified within the N terminus. The C-terminal tail of AE1 is composed of 36 residues; this domain includes three reported DRTA-causing mutations: A888L, 889X,15 M909T,16 and R901X.17 R901X and M909T mutant proteins expressed in Xenopus oocytes exhibit preserved anion transport function, but both are mistargeted to the apical membrane in polarized Madin-Darby canine kidney (MDCK) cells.5,6,16 As a consequence, we and others have sought identification of novel binding partners for this domain, to ascertain molecular basis of sorting, trafficking, and membrane retention of kAE1. Several proteins are reported to interact with the C terminus of kAE1, and disruption of these interactions results in abnormal cellular location of kAE1.18–20

We report here the identification and characterization of a novel C-terminal binding partner, the β1 subunit of the Na+,K+–ATPase for AE1. The sodium pump (Na+,K+–ATPase) is a ubiquitous plasma membrane ATP-requiring enzyme that transports 3 Na+ in exchange for 2 K+. In the kidney, the Na+,K+–ATPase is a major driver of vectorial transport, which plays a crucial role in kidney function.21,22 The minimal functional unit of this pump is composed of α2α and β2β subunits in a probable 1:1 molar ratio. To date, four α (α1–α4) and three β (β1–β3) polypeptides have been identified, of which both α1 and β1 subunits are highly expressed in the kidney.23–25 The α subunit plays a major role in catalytic function of the enzyme, whereas the β subunit appears to be crucial for proper maturation, delivery, and insertion of the whole enzyme into the plasma membrane and also functions to regulate its catalytic activity.26,27 The β subunit is a type II membrane protein with a short cytoplasmic N terminus, single transmembrane helix, and large extracellular domain.

RESULTS

β1 Identified as a Potential Binding Partner for AE1C

Five of 40 positive clones sequenced from a yeast two-hybrid assay using wild-type AE1C (AE1C-WT) as bait to screen a human kidney cDNA library were 100% identical to the coding sequence for the human β1 subunit of Na+,K+–ATPase. A specific yeast cell-mating assay confirmed interactions between AE1C-WT and the β1 containing clones (Figure 1A).

β1 and kAE1 Are in the Same Complex at the Basolateral Membrane of α-ICs in Human Kidney

Dual-immunostaining using anti-AE1(rab) and anti-β1(m) antibodies showed similar basolateral distribution of the two proteins in α-ICs in the collecting duct (Figure 1B). Previously reported glomerular staining of kAE120 was not detected, possibly owing to methodologic or antibody differences. As expected, in contrast with the restricted IC location of kAE1, β1 was also extensively distributed basolaterally throughout the nephron.

To investigate whether the observed colocalization is due to the two proteins being close enough to be within the same complex, communoprecipitation was performed from human kidney cortical tissue membrane fractions where no erythrocyte AE1 was detectable.18 Using anti-β1(rab) as the precipitating antibody (Figure 1C, left, Supplemental Figure 1), a band at approximately 100 kD was detected by Bric170 (anti-AE1) and a similar size of band was detected by anti-α1, confirming presence of both kAE1 and the α1 subunit of Na+,K+–ATPase. Replacing anti-β1(rab) with Bric170 (Figure 1C, right, Supplemental Figure 1) as the precipitating antibody, both β1 and α1 were detectable in the precipitates. Specificity of these assays was confirmed by absence of the communoprecipitated proteins when a negative control IgG was applied in parallel in the assays. All of these data demonstrate close integration between kAE1 and the sodium pump.

Direct Binding of β1N to AE1C Confirmed by ELISA and Fluorescence Titration

Because the AE1C domain and the N terminus of β1 (β1N) both lie intracellularly, we made glutathione S-transferase (GST)-tagged β1N and AE1C-WT proteins (Supplemental Figure 2) and used ELISA to titrate their binding. As shown in Figure 2A, AE1C-WT interacted directly with β1N in a concentration-dependent and saturable manner; this was true whether the GST tag was present on AE1C (latter not shown). Background signals from GST alone were significantly low, confirming specificity, and GST-associated presentation of β1 as a homo-tetramer likely does not contribute to the detected interaction.

The binding affinity of the AE1C-WT/β1N interaction was determined using fluorescence titration (Figure 2B). In contrast with the β1N domain, no Trp residues are present in the C terminus of AE1. AE1C-WT has a single tyrosine, which has negligible fluorescence under these conditions. The β1N Trp fluorescence change observed was dependent on the amount of AE1C-WT, because there was no change detectable when buffer alone was added (data not shown). Upon saturation of binding of the AE1C-WT peptide, there was an approximately 40% decrease in fluorescence change of β1N. Quenching of
N Trp fluorescence yielded a $K_d$ (or $K_{1/2}$) value of 0.81±0.04 μM for the AE1C-WT/b1N interaction.

The Last 11 Residues of AE1 Are Important for β1 Binding in the Human Kidney

Because truncation of the last 11 residues of AE1C causes human dRTA, we were curious whether loss of this subdomain would affect β1 binding. To investigate this, parallel GST pull-down analyses were performed using AE1C-WT or AE1C-Δ11 GST fusion protein incubated with human kidney membrane samples. Precipitated proteins were probed with anti-β1(rab) antibody (Figure 2C) in three separate replicate experiments. Densitometric analysis (Figure 2D) demonstrated an approximately 60% reduction in the amount of β1 pulled down by AE1C-Δ11 mutant compared with WT ($P<0.001$ versus WT), indicating that these 11 residues are essential for a complete binding site for β1, and/or form part of that site.

The β1 Subunit Is Required for Wild-Type Membrane Levels of kAE1 in Kidney Cells

Because native intercalated cell lines are unavailable and HEK293, MDCK, and LLC-PK1 cell lines do not express endogenous kAE1, we have established stable kAE1-expressing lines. Confocal microscopy (Figure 3A) demonstrated green fluorescent protein (GFP)–tagged kAE1 located evenly on the plasma membrane of HEK-pMEP-eGFP-kAE1 cells, and uniquely at the basolateral surface in both polarized MDCK-ΔpMEP-eGFP-kAE1 and LLC-PK1-ΔpMEP-eGFP-kAE1 cells. The β1 subunit of Na+,K+-ATPase is known to be important in maintaining the structure and function of polarized kidney epithelial cells.30 Because disruption of the last 11 residues of AE1 causes AE1 mis-targeting5,6 and reduced β1 binding, we assessed a possible role for β1 in kAE1 membrane targeting/retention in kidney cells. Small interfering RNA (siRNA)–induced knockdown of endogenous β1 in HEK-ΔpMEP-eGFP-kAE1 cells was achieved to at least 90% (Figure 3B, Supplemental Figure 3A); as expected, the level of the endogenous α1 subunit of Na+,K+-ATPase on the plasma membrane was also reduced in the knockdown cells. Similarly, a remarkable decrease of cell surface kAE1 to approximately 50% was observed in the knockdown cells, indicating a requirement of β1 for plasma membrane residency of kAE1.

Conversely, induction of kAE1 expression in MDCK-ΔpMEP-eGFP-kAE1 or LLC-PK1-ΔpMEP-eGFP-kAE1 cells was accompanied by significantly increased levels of β1 (by 60%–100%) on the cell surface in both cell lines (Figure 3C, Supplemental Figure 3B). Levels of cell surface α1 subunit rose concomitantly, indicating increased presence of the whole pump.

β1-Dependent Internalization of kAE1 in Kidney Epithelial Cells

It has been reported that treatment of LLC-PK1 but not MDCK cells with low-dose ouabain leads to internalization of the Na+, K+-ATPase.31 We asked whether kAE1 would be cointernalized upon such treatment. To investigate this, LLC-PK1-ΔpMEP-eGFP-kAE1 cells were treated with 50 nM ouabain.

Figure 1. Identification of the β1 sodium pump subunit as a potential binding partner for kAE1. (A) After yeast two-hybrid screening of a human kidney cDNA library using AE1C-WT as bait, five clones (one shown) corresponding to the β1 subunit of Na+,K+-ATPase display survival of progeny from mating with AE1C-WT containing cells (AE1C-WT), but not with a negative control strain (control). (B) Immunostaining of normal human kidney cortex (lower panels at high power) shows kAE1 (green; a and d) basolaterally in intercalated cells. β1 (red; b and e) distribution is also basolateral but more widespread, colocalizing with kAE1 in collecting ducts (yellow; c and f). (C) Solubilized membrane protein fractions of human kidney are immunoprecipitated using either anti-β1(rab) or Bric170 as indicated. Detection of kAE1 and both β1 and α1 subunits of the sodium pump indicates coimmunoprecipitation. Replacement of the precipitating antibodies with appropriate purified IgGs provided negative controls. Bar, 10 μm.
followed by surface biotinylation to isolate plasma membrane proteins for Western blot analysis. Consistent with the findings of Liu et al., the level of β1 on the plasma membrane was significantly reduced in ouabain-treated cells compared with controls, to about 50% (Figure 3D, Supplemental Figure 3C). Strikingly, the amount of kAE1 on the plasma membrane was significantly reduced in ouabain-treated cells compared with controls, to about 50% (Figure 3D, Supplemental Figure 3C).

Figure 2. In vitro confirmation of direct interaction between AE1 and β1. (A) ELISA plates coated with GST_β1N (filled circles) or GST alone (gray squares) are incubated with increasing concentrations of GST_AE1C-WT followed by detection with anti-AE1(rab). Specific binding of AE1C-WT to β1N is shown. (B) Tag-free β1N is titrated with the increasing concentration of tag-free AE1C-WT using fluorescence titration, yielding saturable binding with $K_D = 0.81 \pm 0.04 \, \mu M$. (C) GST-tagged AE1C-WT or AE1C-Δ11 mutant or GST alone are utilized in bead-bound pull-down assays against solubilized human kidney membrane fractions. Bound proteins are analyzed using anti-β1 (upper blot), with GST blotting providing a loading control (lower blot). Blots are representative of three separate assays. (D) Bands are densitometrically quantified, expressed relative to WT (100%) ± SEM, and subjected to ANOVA analysis, demonstrating the importance of the last 11 residues of AE1 for β1 binding.

**DISCUSSION**

We have shown physical and functional links between kAE1 and the β1 subunit of Na⁺,K⁺-ATPase, after an initial yeast two-hybrid screen. The physiologic relevance of this interaction in mammalian kidney was demonstrated by colocalization and coimmunoprecipitation. The latter included the Na⁺, K⁺-ATPase α1 subunit, suggesting the whole sodium pump to be bound in the kAE1-containing complex.

A prediction for the potential interaction between kAE1 and Na⁺,K⁺-ATPase in rabbit kidney was in fact made 2 decades ago. In that study, Janoshazi et al. noticed that specific inhibition of Na⁺,K⁺-ATPase activity also reduced kAE1’s anion exchange activity in medullary collecting duct cells. The authors hypothesized that the two membrane transporters are close enough together that conformational information can be exchanged between them. However, no further evidence for a direct association has been offered to date. Our ELISA and fluorescence titration assays confirm direct binding between the AE1 C terminus and the β1 N terminus; both domains lie intracellularly when the two proteins are membrane resident. In addition, fluorescence titration indicates a relatively strong affinity between the two proteins. Taken together, these data demonstrate β1 as a novel kAE1 binding partner, and suggest stability of the sodium pump/anion exchange complex.

The importance of the last 11 residues of AE1 in β1 binding is particularly interesting because although anion exchange activity is maintained by truncated AE1, the mutant protein is mistargeted in kidney epithelial cells, implying the presence of basolateral targeting information for kAE1 within the C terminus. Looking in more detail in this region, D902EYDE has been identified as a glyceraldehyde-3-phosphate dehydrogenase binding site, and depletion of endogenous


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glyceraldehyde-3-phosphate dehydrogenase in MDCK cells resulted in defective trafficking of kAE1. The other two previously recognized potential trafficking motifs are adaptor-protein complex μ subunit-binding motif YXXØ (Y904DEV) and a possible class II PDZ-binding domain (A908MPV). Binding of μ subunits, including AP-1B (μ1b), to YXXØ motifs are able to mediate basolateral sorting of certain proteins in epithelial cells. However, both kAE1 and the Na⁺,K⁺-ATPase are sorted to the basolateral surface in the μ1b-deficient cell line LLC-PK1, as is kAE1 in MDCK cells in which either μ1b expression has been suppressed or Val907 (O) in the YXXØ motif has been mutated. AP-1A (μ1a) was recently reported to interact with kAE1 via Y904DEV, and knockdown of endogenous μ1a in HEK293T cells resulted in reduced membrane localization of kAE1. It will be interesting to see whether this is also true in a polarized epithelial system. kAE1’s Y904 is thought to be crucial, because phosphorylation of this residue results in internalization of kAE1 in MDCK cells. Furthermore, we recently identified a novel dRTA-causing mutation, kAE1-M909T, which lies within the potential class II PDZ-binding domain (A908MPV) and converts it to class I (A908TPV). The M909T mutant protein showed a nonpolarized pattern, and deletion of the A908 MPV motif resulted in intracellular retention of the kAE1-Δ4 mutant protein in MDCK cells, indicating that this motif is critical for normal basolateral localization of kAE1. However, the actual PDZ proteins involved are not known. On the basis of these data, it is very likely that there are multiple sorting motifs that determine kAE1’s membrane trafficking, retention, and recycling.

Given the importance of the last 11 residues of kAE1 for both β1 binding and normal membrane residency of kAE1 in kidney epithelial cells, we would have liked to directly to test the hypothesis that β1 binds to kAE1 to help it reach and remain in its normal position. MDCK cells provide an attractive model for studying epithelial protein trafficking because of their clear apicobasolateral polarity and well defined cell junctions. However, depletion of β1 completely disrupts MDCK cell polarity, precluding siRNA experiments in our AE1-expressing MDCK cell lines. Performing these experiments instead in HEK cells resulted in significantly reduced levels of kAE1 on the cell surface, indicating a requirement for β1 for kAE1 membrane destination and/or retention. In addition, overexpression of plasma membrane–located kAE1 in both MDCK and LLC-PK1 cells led to much higher levels of β1 on the cell surface compared with cells without kAE1. Therefore, kAE1 is clearly not essential for normal membrane location of β1 in either cell type, β1 is either behaving as a chaperone for kAE1 or is stabilizing it at the plasma membrane. The latter possibility is supported by our internalization assays, in which ouabain treatment cointernalized both proteins.

An attractive possible mechanism for cointernalization comes from the knowledge that ouabain activates Src kinases, which because of the close proximity of the sodium pump and AE1 could result in phosphorylation of both. However, our experimental system precluded directly testing this hypothesis, because published methods require some serum starvation to see an effect of Src kinase inhibition in LLC-PK1 cells, which our cells did not survive when serum starvation and kAE1 expression were carried out simultaneously, and omitting this step abolished the effects of that inhibition (data not shown).

AE1’s linkage to the underlying actin cytoskeleton is well recognized, but the nature of this has remained uncertain. In
weakening the hypothesis.\textsuperscript{41} Much earlier, double-immunolabeling studies demonstrated colocalization between kAE1 and ANK basolaterally in \(\alpha\)-ICs in rat kidney, suggesting that ankyrin might be the linker.\textsuperscript{45} However, unlike the longer eAE1, the N terminus of kAE1 did not show direct interaction with erythroid ANK1 in \textit{in vitro} assays.\textsuperscript{46,47} It has since become evident that ANK3 (not ANK1) is the kidney isoform, but there is no separate evidence to suggest that ANK3 interacts with kAE1, probably because of structural differences between the N-terminal cytosolic domains of eAE1 and kAE1.\textsuperscript{14} In the case of the Na\(^{+}\),K\(^{+}\)-ATPase, its \(\alpha\) subunit is linked to the cytoskeleton through ANK, including ANK3, which is involved in targeting and stabilization of the pump at the plasma membrane.\textsuperscript{48–51} Thus, a direct link between kAE1 and the sodium pump can provide the indirect link between kAE1 and cytoskeleton through the pump.

As we have shown, another potential explanation for parallel increases of \(\beta 1\) on the plasma membrane and increased surface levels of kAE1 is that of linked adjustment between activities of the two transporters. The amount of sodium pump on the plasma membrane is directly related to total activity of the pump in cells, as shown by Geering \textit{et al.}\textsuperscript{52} and Liu \textit{et al.}\textsuperscript{31} The former showed increased pump activity when expression levels rose in \textit{Xenopus} oocytes, whereas the latter showed that ouabain-induced internalization of Na\(^{+}\),K\(^{+}\)-ATPase reduced levels of both \(\alpha\)1 and \(\beta\)1 subunits and total activity of the enzyme in parallel. Inhibition of the sodium pump also results in reduction in both sodium reabsorption and potassium secretion.\textsuperscript{21,53} Therefore, when there is more membrane-bound Na\(^{+}\),K\(^{+}\)-ATPase, and hence increased cation exchange activity, the local/neighboring cellular ionic environment may be altered and kAE1 activity may be upregulated, and \textit{vice versa}. In support of the latter, \(\alpha E1^{-/-}\) mouse kidney tissue sections revealed significantly less \(\beta 1\) staining on the plasma membrane not only of \(\alpha\)-ICs, but also of principal cells in the collecting duct, compared with \(\alpha E1^{+/+}\) mice. In kidney epithelial cell lines, \(\beta 1\) looks normal in the absence of kAE1. However, distribution of \(\beta 1\) is much more widespread than that of AE1; thus, the generally decreased levels of membrane-bound \(\beta 1\) observed in the \(\alpha E1^{-/-}\) mouse cannot be explained simply by loss of physical interaction of the two proteins. Indeed, patients with dRTA, which is caused by defective \(\beta 1\)-IC function, are somewhat salt and volume depleted, with secondary hyperaldosteronism. Taken together, these findings are consistent with a paracrine regulatory role for AE1.

In conclusion, we have identified a direct interaction between kAE1 and the \(\beta 1\) subunit of the sodium pump, and our data introduce \(\beta 1\) not only as a novel binding partner for the C-terminal tail of AE1 but also as a component of kAE1’s ability to achieve and retain basolateral membrane residency in kidney cells. Identification of the \(\beta 1\)/AE1 interaction indicates a direct link, and a potential regulatory interaction, between important anion and cation transporters in the kidney.

Table 1. Biochemistry after dietary acidification of 3-month-old wild-type C57BL/6J mice

<table>
<thead>
<tr>
<th>Blood</th>
<th>Chronically Acidified</th>
<th>Controls</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.3±0.03</td>
<td>7.4±0.04</td>
<td>NS</td>
</tr>
<tr>
<td>HCO(_3)^{-}</td>
<td>18.5±0.81</td>
<td>20.7±0.52</td>
<td>0.05</td>
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<tr>
<td>pCO(_2)</td>
<td>5.1±0.45</td>
<td>4.9±0.43</td>
<td>NS</td>
</tr>
<tr>
<td>Urine</td>
<td>pH 5.8±0.18</td>
<td>6.6±0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>FE Na(^{+}) (%)</td>
<td>0.48±0.04</td>
<td>0.48±0.03</td>
<td>NS</td>
</tr>
<tr>
<td>FE K(^{+}) (%)</td>
<td>28.0±3.12</td>
<td>27.0±4.70</td>
<td>NS</td>
</tr>
</tbody>
</table>

All values are mean±SEM. Acidified animals (n=6) developed a compensated metabolic acidosis, with blood pH and pCO\(_2\) concentrations remaining similar compared with controls, which is consistent with a previous report.\textsuperscript{59}
Antisera

The following antibodies were used in this study: anti-AE1 (rabbit polyclonal; gift from Dr. E. Martinez-Anso, Pamplona, Spain) and BrC-170 (mouse monoclonal anti-AE1; IBGRL, 9540); anti-α1 and anti-β1 (mouse monoclonals, recognizing α1/β1 subunits of Na⁺, K⁺-ATPase, respectively, A-276, A-278; Sigma-Aldrich), anti-β1 (rabbit polyclonal, recognizes the β1 subunit of Na⁺, K⁺-ATPase, ab96292; Abcam, Inc.), anti-AQP2 (goat polyclonal, recognizes Aquaporin2, sc-9882; Santa Cruz Biotechnology), anti-B1 and α4 (rabbit polyclonals, recognize B1/α4 subunit of H⁺-ATPase, respectively), anti-E-Cadherin (mouse monoclonal, recognizes E-Cadherin, 610181; BD Transduction Laboratories), anti-α-tubulin (mouse monoclonal, T9026; Sigma-Aldrich), anti-GST (goat polyclonal, 27-4577-01; Cell Signaling Technology) were used in Western blot analysis and immunochemistry, respectively.

Species-specific horseradish peroxidase–conjugated (Dako) and NorthernLight-493 or NorthernLight-557 (R&D Systems) secondary antibodies were used in Western blot analysis and immunochimistry, respectively.

Purified rabbit IgG (15006; Sigma-Aldrich) or mouse IgG1 (G3A1, 5415; Cell Signaling Technology) were used in immunoprecipitation assays as negative controls.

Expression Constructs

To express AE1 C-terminal or β1 N-terminal domain in Escherichia coli, cDNA encoding the last 36 residues of AE1 (AE1C or AE1C-WT) (–L876IFRNVELQCLDADDAKATFDEEEGRDEYDEVAMPV911), or truncating the last 11 residues of the AE1C (AE1C-Δ11), or the first 31 residues of the Na⁺, K⁺-ATPase β1 subunit (β1N) (M1ARGKAKEEGSWKKFIWNSEKKEFLGRTGGS31–) was cloned into pGEX-4T-1 vector to create N-terminal GST-tagged AE1C-WT, AE1C-Δ11, or β1N constructs.

To express intact kAE1 in mammalian cells, full-length cDNA was cloned into a zinc-inducible vector, ΔpMEP4, to create N-terminal eGFP-tagged kAE1 (ΔpMEP-eGFP-kAE1) as previously described. All inserts were amplified in-house by PCR and sequence-verified before use.

Protein Expression and Purification in E. coli Cells

GST-tagged AE1C-WT (GST_AE1C-WT), AE1C-Δ11 mutant (GST_AE1C-Δ11), or β1N (GST_β1N) fusion proteins were expressed in E. coli BL21 cells and purified using glutathione Sepharose beads. To remove the GST tag from GST_AE1C-WT and GST_β1N, each purified fusion protein was first incubated with thrombin and tag-free AE1C-WT or β1N were HPLC purified as previously reported.

Plasmid or siRNA Transfection and Cell Culture

Cells were grown and maintained in DMEM (Sigma-Aldrich) supplemented with FBS (10%), penicillin (100 U/ml)/streptomycin (100 μg/ml), and l-glutamine (2 mM) at 37°C with 95% air and 5% CO₂. HEK293, MDCKII, and LLC-PK1 all express endogenous Na⁺, K⁺-ATPase, but not AE1. To overexpress kAE1, we transfected HEK293, MDCKII, and LLC-PK1 with ΔpMEP-GFP-kAE1 using FuGENE 6 transfection reagent (Roche), the Cell Line Nucleofection Kit L (Axama), and Lipofectamine transfection reagent (Invitrogen), respectively. After selection for 2–3 weeks with Hygromycin B (200 μg/ml), cells were FACS sorted for GFP fluorescence. Sorted cells were further selected to derive mixed or single stable clones and maintained in media containing Hygromycin B, and GFP-kAE1 expression was monitored regularly by immunofluorescence and Western blot analysis.

Endogenous Na⁺, K⁺-ATPase β1 subunit expression in stable HEK-ΔpMEP-eGFP-kAE1 cells was depleted with a siRNA oligonucleotide (ID s1735; Ambion) specifically targeting human ATP1B1. The oligonucleotide, or a nonspecific siRNA (Silencer Negative Control No. 1 siRNA; Ambion) were transfected into the cells at a final concentration of 30 nM using Lipofectamine RNAiMAX reagents (Invitrogen) according to the manufacturer’s instructions. Analysis was conducted 48 hours later.

Yeast Two-Hybrid Assay

Yeast two-hybrid screening was carried out using AE1C-WT as a bait protein to screen the Pretransformed Human Kidney Matchmaker cDNA Library (Clontech) as previously described. Positive colonies were verified by specific yeast mating tests, and were then sequenced and identified through BLAST searching (http://ncbi.nlm.gov/blast).

Immunofluorescence Microscopy

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. Kidneys of untreated Slc4a1−/− (Ae1−/−)32 and wild-type Slc4a1+/+ (Ae1+/+) mice (on a C57BL/6 background) were kindly gifted by Dr. Carsten Wagner (Switzerland). Kidneys were also obtained from C57BL/6J mice rendered acidic by chronic dietary NH₄Cl loading.

Kidney sections (5 μm thick) were fixed in formaldehyde (4%) and embedded in paraffin wax, and were then dual-immunostained for Br1 and either kAE1, Aquaporin2, ZO-1, E-Cadherin, H⁺-ATPase B1, or α4 subunit using previously described methods. Antigen retrieval was carried out using either Proteinase-K (50 μg/ml) for 4 minutes at room temperature or using a pressure cooker containing 0.1 M sodium citrate buffer (pH 6.0) for 2 minutes. After blocking with 10% FBS in PBS/0.01% Tween 20 (blocking buffer), primary antibodies at 1:50 dilution in blocking buffer were applied and sections were incubated at 4°C overnight. Fluorochrome-conjugated secondary antibodies were all used at 1:100 dilution for 1 hour at

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room temperature. Sections were mounted in Vectashield Mounting Medium (Vector Laboratories) and were examined with a Leica SPE confocal laser scanning microscope (Leica Microsystems). As controls, primary antibodies were replaced by either nonimmune serum or isotype-specific antisera and all steps were followed unchanged.

Cell Surface Biotinylation
To examine levels of KAII or Na\(^+\),K\(^+\)-ATPase at the plasma membrane, cell surface biotinylation was performed using the Cell Surface Protein Isolation Kit (Pierce) according to the manufacturer’s instructions. Cells were lysed in buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, 10% glycerol, 1.5% n-Nonyl-\(\beta\)-D-glucopyranoside (n-NDG), 250 mM NaCl, and PIC. Five micrograms each of anti-\(\beta_1\)(m) or rabbit IgG, or 10 \(\mu\)g of either Bric170 or mouse IgG1, were bound to protein G-Sepharose beads before overnight incubation with solubilized human kidney membrane fraction at 4°C. GST immobilized onto glutathione Sepharose beads, followed by incubation overnight with the recovered kidney supernatants. After extensive washing with PBS+1% Triton-X-100 and horseradish peroxidase conjugated antibodies and signals were detected with anti-AE1(rab) antibody, and band intensity was measured using ImageJ software (National Institutes of Health).

ELISA Analyses
ELISA was performed as previously described\(^{18}\) by immobilizing 35 \(\mu\)M GST-B1N or GST alone onto a 96-well plate and incubating with GST-AE1C-WT (0.05–6 \(\mu\)g/ml). Detection was with anti-AE1(rab) and horseradish peroxidase-conjugated antibodies and signals were visualized with 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

Binding Affinity Study
Fluorescence titration was performed in a LS55 Luminescence spectrometer (PerkinElmer) at 25°C. Excitation was set at 295 nm and emission at 340 nm for tryptophan residues in \(\beta_1\)N. \(\beta_1\)N (3 \(\mu\)M) was titrated with AE1C-WT protein over the range from 0.16 to 7.17 \(\mu\)M. Both \(\beta_1\)N and AE1C-WT are GST tag free and were HPLC purified (approximately 95% pure) before use. After each addition, the solution was allowed to equilibrate for 1 minute before recording Trp fluorescence emission. Quenching of Trp fluorescence was analyzed using the \(F_0/F\) ratio (where \(F_0\) and \(F\) are the fluorescence intensities at 340 nm in the absence and presence of AE1C-WT, respectively) plotted against the AE1C-WT concentration using Origin software (OriginLab). The inner filter effect is negligible under the experimental conditions used. The assay was repeated three times.

Internalization Assay
Ouabain-induced internalization of \(\beta_1\) subunit of Na\(^+\),K\(^+\)-ATPase assay was carried out as described.\(^{31}\) Stable LLC-PK1-\(\Delta\)pMEP-eGFP-KAII or MDCK\(\Delta\)pMEP-eGFP-KAII cells seeded onto Transwell filters were grown for 6 and 4 days, respectively. Cells were then serum starved for 12 h before incubation with 50 nM ouabain for 12 hours. The ouabain-treated cells were cell surface biotinylated and membrane protein fractions were immunoblotted with anti-\(\beta_1\)(m) and Bric170 antibodies. Absence of ouabain served as a control.

Acidification
C57Bl/6 wild-type mice (n=6) from age 2.5 months had their drinking water supplemented with either 0.28 M NH\(_4\)Cl/2% sucrose or 2% sucrose alone (controls). After at least 17 days of treatment, pairs of animals were transferred to metabolic cages for urine collection, after which they were terminally anesthetized and blood was collected by cardiac puncture. Blood and urine analyses were carried out as described by Norgett et al.\(^{57}\) The FE values of sodium (FE Na\(^+\)) and potassium (FE K\(^+\)) were calculated from the measured values. All procedures conformed to the 1986 UK Home Office Animals Scientific Procedures Act.

Statistical Analyses
Statistical analyses were performed using either unpaired t tests or ANOVA.

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DISCLOSURES
None.

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