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ACID-BASE REGULATION

# Role of nonconserved charged residues of the AE2 transmembrane domain in regulation of anion exchange by pH

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**Abstract** The ubiquitous AE2/SLC4A2 anion exchanger is acutely and independently regulated by intracellular ($pH_i$) and extracellular pH ($pH_o$), whereas the closely related AE1/SLC4A1 of the red cell and renal intercalated cell is relatively pH-insensitive. We have investigated the contribution of nonconserved charged residues within the C-terminal transmembrane domain (TMD) of AE2 to regulation by pH through mutation to the corresponding AE1 residues. AE2-mediated $Cl^-/Cl^-$ exchange was measured as 4,4′-di-isothiocyanatostilbene-2,2′-disulfonic acid-sensitive $^{36}Cl^-$ efflux from *Xenopus* oocytes by varying $pH_i$ at constant $pH_o$, and by varying $pH_o$ at near-constant $pH_i$. All mutations of nonconserved charged residues of the AE2 TMD yielded functional protein, but mutations of some conserved charged residues (R789E, R1056A, R1134C) reduced or abolished function. Individual mutation of AE2 TMD residues R921, F922, P1077, and R1107 exhibited reduced $pH_i$ sensitivity compared to wt AE2, whereas TMD mutants K1153R, R1155K, R1202L displayed enhanced sensitivity to acidic $pH_i$. In addition, $pH_o$ sensitivity was significantly acid-shifted when nonconserved AE2 TMD residues E981, K982, and D1075 were individually converted to the corresponding AE1 residues. These results demonstrate that multiple conserved charged residues are important for basal transport function of AE2 and that certain nonconserved charged residues of the AE2 TMD are essential for wild-type regulation of anion exchange by $pH_i$ and $pH_o$.

**Keywords** Nonconserved charged residues · Anion exchange · *Xenopus* oocytes

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## Introduction

In mammalian cells, $Na^+$-independent $Cl^-/HCO_3^-$ exchangers encoded by members of the SLC4 and SLC26 gene superfamilies contribute to the maintenance of cellular pH, volume, and intracellular chloride concentration. Polypeptide products of at least three homologous SLC4 genes, AE1/SLC4A1, AE2/SLC4A2, and AE3/SLC4A3, are differentially expressed in various cells and tissues and mediate electroneutral anion exchange [4, 29]. AE1 polypeptides are expressed at high level in erythrocytes and in Type A intercalated cells of the renal collecting duct, whereas AE2 and AE3 are widely expressed in numerous tissues. SLC4/AE-mediated anion exchanger gene products differ in acute regulation by intracellular pH ($pH_i$) and extracellular pH ($pH_o$). AE1-mediated anion exchange in erythrocytes [16] and *Xenopus* oocytes [20, 38] is relatively insensitive to changes in pH across the physiological range. In contrast, AE2- and AE3-mediated $Cl^-/HCO_3^-$ exchange has been shown to be acutely regulated by changes in pH [32–34]. Mice genetically engineered to lack AE2 are runted, achlorhydric, fail to undergo tooth eruption, and die before weaning [17].

The AE polypeptides have highly conserved hydrophobic transmembrane domains (TMD) and less extensively conserved hydrophilic N-terminal cytoplasmic domains. The C-terminal TMD suffices to mediate anion exchange in the absence of nearly the entire N-terminal cytoplasmic domain and is predicted to span the lipid bilayer 12–14 times [18, 23, 24]. The pH-sensitive properties of AE2 have been localized to both the N-terminal cytoplasmic domain and the C-terminal TMD. We have previously identified two noncontiguous regions within the AE2 N-terminal cytoplasmic domain that are involved in regulation of AE2 by pH [32–34]. Some of these residues are conserved in the AE1 N-terminal cytoplasmic domain, but investigation of the role of TMD residues in the regulation of AE2-mediated anion exchange by pH has been less extensive. Our initial work has shown that both conserved and nonconserved AE2 TMD histidine residues, although essential for basal levels of $Cl^-$ transport, contribute to but are not sufficient to explain wild-type regulation of AE2 by pH [35]. We hypothesized that charged residues of the AE2 C-terminal TMD not conserved in the corresponding positions of the AE1 TMD also contribute to the regulation of AE2-mediated $Cl^-$/base exchange by pH. In the current study, we characterize the involvement of nonconserved acidic and basic amino acid residues of the C-terminal TMD, as well as selected nonconserved uncharged residues, in pH-dependent regulation of AE2 activity. We identify distinct groups of amino acids in the TMD whose mutation selectively alters regulation of AE2 by $pH_i$ and $pH_o$.

## Materials and methods

### Materials

Chemical reagents of analytical grade were purchased from Sigma, Fluka (St Louis, MO), or Calbiochem (San Diego, CA). $Na^{36}Cl$ was obtained from ICN (Irvine, CA). Taq DNA polymerase was from Roche Biochemicals (Mannheim, Germany), dNTPs were from Promega (Madison, WI). Restriction enzymes and T4 DNA ligase were from New England BioLabs (Beverly, MA).

### Construction of mutant mAE2 cDNAs

Plasmid pΔX [3] encoding mouse AE2 (Accession: J04036) served as the cDNA template for polymerase chain reaction (PCR). Single codon mutations (or as indicated double codon mutations) were introduced into the AE2 TMD coding region by a four primer PCR method described previously [9, 32, 38] and inserted in the oocyte expression vector pXT7 [13]. Integrity of all PCR fragments and ligation sites was confirmed by DNA sequencing of both strands. Oligonucleotides were purchased from Biosynthesis (Woodlands, TX). Primer sequences are available upon request.

### cRNA expression in *Xenopus* oocytes

Ovarian segments from female *Xenopus laevis* (Xenopus One, Madison, WI) anesthetized with 0.17% tricaine according to protocols approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center were excised, minced, and incubated in 2 mg/ml Type A collagenase (Roche) for 1 h at room temperature in ND-96, pH 7.4, containing (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, 2.5 sodium pyruvate, and 5 mg/100 ml gentamicin. Stage V–VI oocytes were manually defolliculated and injected on the same day with 50 nl of cRNA or with $H_2O$. Capped mAE2 cRNAs were transcribed from linearized cDNA templates with the T7 MEGAscript kit (Ambion, Austin, TX), purified with the RNAeasy kit (Qiagen, Valencia, CA), and resuspended in diethylpyrocarbonate-treated water. Formaldehyde agarose gel electrophoresis was used to test cRNA integrity, and cRNA concentration was estimated by $A_{260}$ (Nanodrop®, Wilmington, DE). The amount of cRNA injected per mutant AE2 (0.5–25 ng) was titrated to approximate wt AE2-mediated $^{36}Cl^-$ efflux activity at $pH_o$ 7.4 associated with injection of 10 ng wt AE2 cRNA. Injected oocytes were maintained at 19°C in ND-96 for 2–6 days in the continued presence of gentamicin and pyruvate until used for assays.

### $^{36}Cl^-$ efflux assays in *Xenopus* oocytes

$^{36}Cl^-$ efflux assays were performed as previously described [34, 35]. Briefly, oocytes were injected with 50 nl of 260 mM $Na^{36}Cl$ (15,000–20,000 cpm) and allowed to recover 5–10 min in $Cl^-$-free medium (in mM, 96 Na isethionate, 2 K gluconate, 1.8 Ca gluconate, 1 Mg gluconate, 5 Hepes, pH 7.4). $^{36}Cl^-$ efflux was initiated by transferring individual oocytes to 6-ml borosilicate glass tubes containing 1 ml ND-96 (in mM, 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, pH 7.4). At 3-min intervals, 0.95 ml of this efflux solution were removed and replaced by an equal volume of fresh ND-96. At the end of the assay, integrity of AE2-mediated $Cl^-$ transport and of the oocyte was confirmed by a final efflux period in the presence of the anion transport inhibitor 4,4′-di-isothiocyanatostilbene-2,2′-disulfonic acid (200 μM). Individual oocytes were lysed in 100 μl 1% sodium dodecyl sulfate. Lysates and efflux samples were subjected to scintillation counting for 3–4 min such that the magnitude of two SD was <5% of the sample mean.

$^{36}Cl^-$ efflux activity of tested mutant AE2 polypeptides was compared to wt AE2 at $pH_o$ 7.4 on each experimental

day, and water-injected oocytes and AE2 cRNA-injected oocytes from the same frog were tested in parallel measurements within each experiment. Every AE2 mutant was tested in oocytes harvested from at least two frogs. The complete set of $^{36}Cl^-$ efflux data is presented in Supplemental Table 1. Experimental data were plotted as ln (% cpm remaining in the oocyte) vs time. $^{36}Cl^-$ efflux rate constants were calculated from linear fits to data from at least three time points for each experimental condition. Measurements of $pH_o$-dependent AE2-mediated $^{36}Cl^-$ efflux and of $pH_i$-dependent AE2-mediated $^{36}Cl^-$ efflux were conducted as previously described [34]. $^{36}Cl^-$ efflux rate constants for $pH_i$ dependence of AE2 were normalized as the ratio of the efflux rate constant in the presence of 40 mM butyrate to that in the absence of butyrate at constant $pH_o$ 7.4. As shown previously, butyrate is neither a substrate nor an inhibitor of AE2 [32].

### *Confocal laser immunofluorescence microscopy*

The level of expression of functionally inactive AE2 TMD mutants at or near the oocyte surface was assessed in oocytes expressing AE2 mutant carboxy-terminal green fluorescent protein (GFP) fusion polypeptides.

At least eight oocytes expressing each AE2–GFP fusion construct were fixed overnight in phosphate-buffered saline (PBS) containing 3% paraformaldehyde, washed three times in PBS, incubated overnight in 30% sucrose in PBS, then mounted in OCT, and frozen in liquid $N_2$. Twenty-micrometer cryosections were imaged with a BioRad MRC1024 laser scanning confocal microscope. Representative sections imaged at constant laser intensity and filter settings were compiled in Microsoft PowerPoint.

### *Statistical analysis*

Data are reported as mean±SEM. Values for individual mutants, and groups of mutants were compared to those of wild-type AE2 by Dunnett's two-way *t* test. The level of significance was taken as $p<0.05$.

## Results

### Function of AE2 TMD mutants

We have shown that the AE2 C-terminal TMD mediates pH-sensitive anion exchange in the absence of nearly the entire N-terminal cytoplasmic domain [32]. Recently, we reported that AE2 TMD histidine residues are involved in pH regulation, but mutagenesis of these residues does not completely abolish pH sensitivity [35]. We therefore hypothesized that one or more additional critical amino acid residues of the AE2 C-terminal TMD are required for sensing and responding to changes in $pH_i$ and $pH_o$, and that at least some of these residues are not conserved in the relatively pH-insensitive AE1 anion exchanger. Amino acid sequence alignment of mouse AE2 and mouse erythroid AE1 (Fig. 1a) shows the many nonconserved and several conserved residues examined in this study. Figure 1b depicts the putative locations of these conserved and nonconserved amino acid residues within the AE2 TMD modeled on available AE1 topographical data [39]. Most nonconserved charged residues of the AE2 TMD are predicted to reside in intracellular or extracellular loops, but several of those predicted to reside within the plane of the lipid bilayer may be accessible to intracellular and/or extracellular protons (Fig. 1b). We tested the hypothesis that the AE2 TMD pH "sensor" might be a single nonconserved charged residue.

We chose for study 36 residues of the AE2 TMD, 28 of which are not conserved in the corresponding positions in AE1. Thirteen of these nonconserved residues have positively charged side chains, and thirteen are negatively charged. The remaining two residues not conserved in AE1 (AE2 F922 and P1077) are conserved in the corresponding positions of the pH-sensitive, homologous anion exchanger, AE3. The AE2 amino acid residues chosen for mutation were in most cases substituted with the corresponding AE1 residues (Summary Table 1). Charged AE2 residues conserved in AE1 were mutated either to the opposite charge or to the amide, to Ala, to Cys or, in the cases of E771/E772 and E821, to aspartate. The latter three mutations tested the effect of side chain length while maintaining charge. All AE2 TMD mutants were tested for "basal" functional expression in *Xenopus* oocytes defined as $^{36}Cl^-$ efflux at $pH_o$ 7.4.

Figure 2a shows basal activity of the mutants within AE2 aa 703–922, the N-terminal portion of the AE2 TMD encompassing putative TM 1–6. Figure 2b presents basal activity of the mutants within AE2 aa 923–1202, the C-terminal portion of the AE2 TMD, encompassing the remaining 6–8 transmembrane spans. Most mutant AE2 polypeptides exhibited activity similar to that of wild-type AE2. AE2 mutants in residues near the polypeptide's C terminus, E1133D, K1153R, R1155K, and R1202L, exhibited significantly reduced $^{36}Cl$ efflux activity, which nonetheless sufficed to allow analysis of regulation by $pH_o$ and $pH_i$. Three AE2 mutants (R789E, R1056A, R1134C) exhibited no measurable $^{36}Cl^-$ efflux activity in *Xenopus* oocytes and so could not be analyzed further for their impact on transport regulation. Two of these nonfunctional mutants, AE2 R789E and AE2 R1056A, were shown to be present at or near the cell surface (Fig. 2c). Thus, at least some of the loss-of-function mutations retained a conformation competent for trafficking to or towards the cell

**a**

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AE1  423  QVLAAVIFIYFAALSPAVTFGGLLGEKTRNLMGVSELLISTAVQGILFALLGAQPLLVLGFSGPLLVFEEAFFSFCESNNLEYIVGRAWIGFWLILLVML   522
AE2  703  QCLAAVIFIYFAALSPAITFGGLLGEKTKDLIGVSELIMSTALQGVVFCLLGAQPLLVIGFSGPLLVFEEAFFSFCSSNELEYLVGRVWIGFWLVFLALL   802
AE3  706  QCVAAVLFIYFAALSPAITFGGLLGEKTEGLMGVSELIVSTAVLGVLFSLLGAQPLLVVGFSGPLLVFEEAFFKFCRAQDLEYLTGRVWVGLWLVVFVLA   805

AE1  523  VVAFEGSFLVQYISRYTQEIFSFLISLIFIYETFSKLIKIFQDYPL.................QQTYAPVVMKP...........KPQGPVPNTALFS___592
AE2  803  MVALEGSFLVRFVSRFTQEIFAFLISLIFIYETFYKLIKIFQEHPLHGCSGSNDS.EAGSSSSSNMTWATTILVPDNSSASGQSGQEKPRGQ.PNTALLS  900
AE3  806  LVAAEGTFLVRYISPFTQEIFAFLISLIFIYETFHKLYKVFTEHPLLPFYPPDEALETGLELNS......SALPP....TEGPPG...PRNQ.PNTALLS  891

AE1  593  LVLMAGTFLLAMTLRKFKNSTYFPGKLRRVIGDFGVPISILIMVLVDSFIKGTYTQKLSVPDGLKVSNSSARGWVIHPLGLYRLFPTWMMFASVLPALLV   692
AE2  901  LVLMAGTFFIAFFLRKFKNSRFFPGRIRRVIGDFGVPIAILIMVLVDYSIEDTYTQKLSVPSGFSVTAPDKRGWVINPLGEKTPFPVWMMVASLLPAVLV  1000
AE3  892  LILMLGTFLIAFFLRKFRNSRFLGGKARRIIGDFGIPISILVMVLVDYSITDTYTQKLTVPTGLSVTSPHKRTWFIPPLGSARPFPPWMMVAAAVPALLV   991

AE1  693  FILIFLESQITTLIVSKPERKMIKGSGFHLDLLLVVGMGGVAALFGMPWLSATTVRSVTHANALTVMGKASGPGAAAQIQEVKEQRISGLLVSVLVGLSI   792
AE2 1001  FILIFMETQITTLIISKKERMLQKGSGFHLDLLLIVAMGGICALFGLPWLAAATVRSVTHANALTVMSKAVAPGDKPKIQEVKEQRVTGLLVALLVGLSM  1100
AE3  992  LILIFMETQITALIVSQKARRLLKGSGFHLDLLLIGSLGGLCGLFGLPWLTAATVRSVTHVNALTVMRTAIAPGDKPQIQEVREQRVTGVLIASLVGLSI  1091

AE1  793  LMEPILSRIPLAVLFGIFLYMGVTSLSGIQLFDRILLLFKPPKYHPDVPFVKRVKTWRMHLFTGIQIICLAVLWVVKSTPASLALPFVLILTVPLRRLIL   892
AE2 1101  VIGDLLRQIPLAVLFGIFLYMGVTSLNGIQFYERLHLLLMPPKHHPDVTYVKKVRTMRMHLFTALQLLCLALLWAVMSTAASLAFPFILILTVPLRMVVL  1200
AE3 1092  VMGAVLRRIPLAVLFGIFLYMGVTSLSGIQLSQRLLLIFMPAKHHPEQPYVTKVKTWRIDLFTCIQLGCIALLWVVKSTAASLAFPFLLLLTVPLSGCLL  1191

AE1  893  PLIFRELELQCLDGDDAKVTFDEENGLDEYDEVPMPV  929
AE2 1201  TRIFTEREMKCLDANEAEPVFDECEGVDEYNEMPMPV  1237
AE3 1192  PRLFQDRELQALDSEDAEPNFDE.DGQDEYNELHMPV  1227
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**b**

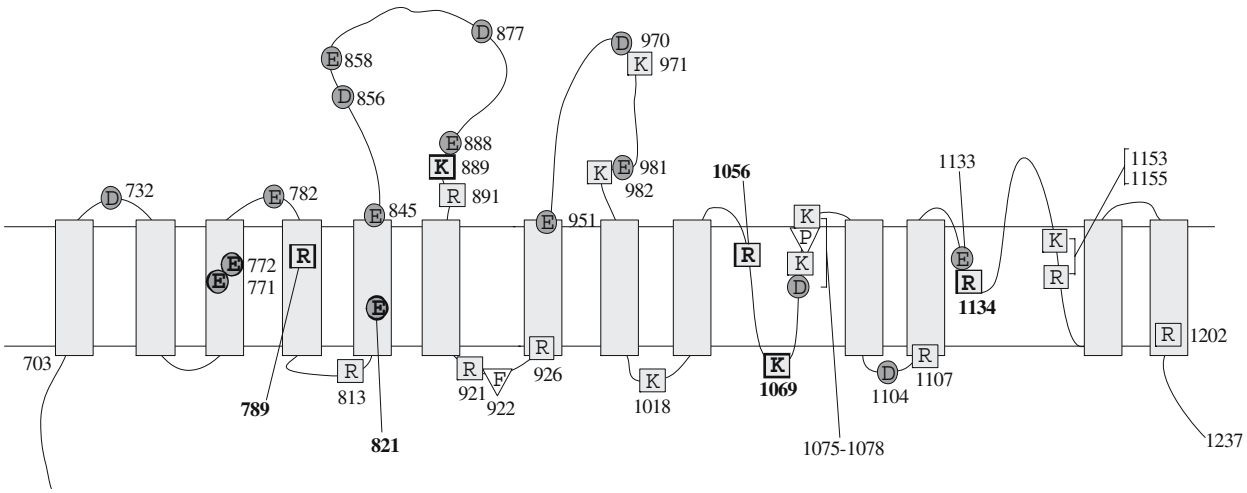


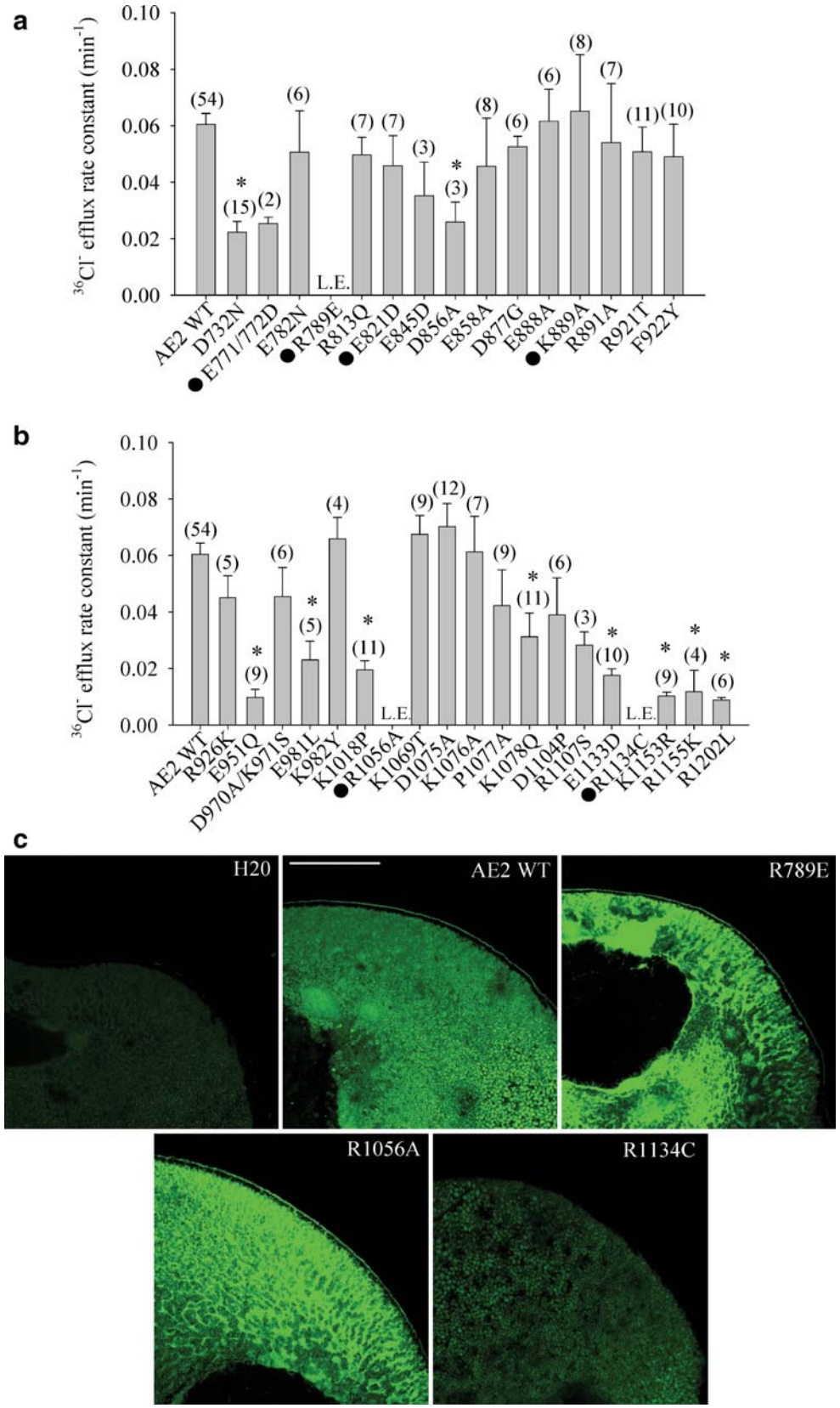

**Fig. 1** Conserved and nonconserved AE2 transmembrane domain amino acids. **a** Aligned amino acid sequences of mouse AE2 (slc4a2: J04036), mouse AE1 (slc4a1; J02756), and mouse AE3 (slc4a3; AAA40692). The *shaded boxes* highlight the nonconserved and conserved residues mutated in the current study. Predicted transmembrane domain spans are underlined in *bold* for AE2. **b** Putative locations in the AE2 TMD of the nonconserved and conserved residues with charged and uncharged side chains mutated in this study, modeled on the AE1 topography proposed by Zhu et al. [40]. Acidic residues are in *dark gray circles*, alkaline residues are in *light gray boxes* and uncharged residues are in *white inverted triangles*. AE2 TMD residues in *bold* are conserved in mAE1

surface of the oocyte but no longer competent for anion transport. In contrast, however, AE2 R1134C was not detected at or near the oocyte surface membrane but was retained intracellularly. Subsequent experiments were restricted to functionally active mutants.

## AE2 TMD residues of putative TM1-6 (aa 703–922) that are nonconserved in AE1 contribute to the regulation of AE2 activity by pH

We have shown previously that clusters of noncontiguous amino acids in the AE2 N-terminal cytoplasmic domain are important for pH sensitivity [34]. We therefore tested first the hypothesis that individual TMD amino acid residues not conserved in AE1 influence the $pH_i$ sensitivity of AE2. Figure 3a (TMD aa 703–922) shows predicted locations of the AE2 TMD mutations tested. Bath addition and subsequent removal of the weak acid, butyrate, was used to decrease and then increase oocyte $pH_i$ [32]. Figure 3b shows efflux rate constants in the presence (black bars) and subsequent removal (gray bars) of 40 mM butyrate measured in oocytes expressing wild-type AE2 or the indicated AE2 mutant polypeptides. As summarized in

**Fig. 2** Charged residues within the AE2 TMD are involved in $Cl^-$ transport. **a** $^{36}Cl^-$ efflux rate constants measured at $pH_o$ 7.4 in (*n*) oocytes expressing wt AE2 or the indicated AE2 TMD mutants in TMs 1–6 (aa 703–922; mean±S.E.). *Asterisk*, $p<0.05$ for indicated comparisons. **b** $^{36}Cl^-$ efflux rate constants measured at $pH_o$ 7.4 in (*n*) oocytes expressing wt AE2 or the indicated AE2 TMD mutants in the C-terminal portion of the TMD (aa 926–1202; mean ± S.E.). *Asterisk*, $p<0.05$ for indicated comparisons. (*L.E.*, low expression; rate constant too low to allow analysis). *Black circles* indicate residues conserved in mAE1. *Asterisk*, $p<0.05$ compared with wt AE2. **c** Representative confocal fluorescence images of cryosections from oocytes previously injected with water or with cRNA encoding the indicated AE2-GFP fusion protein. *Bar*, 180 μm


a
$^{36}Cl^-$ efflux rate constant ($min^{-1}$)
0.10
0.08
0.06
0.04
0.02
0.00
(54)
(15)
(2)
(6)
L.E.
(7)
(7)
(3)
(3)
(8)
(6)
(6)
(8)
(7)
(11)
(10)
AE2 WT
D732N
E771/772D
E782N
R789E
R813Q
E821D
E845D
D856A
E858A
D877G
E888A
K889A
R891A
R921T
F922Y
b
$^{36}Cl^-$ efflux rate constant ($min^{-1}$)
0.10
0.08
0.06
0.04
0.02
0.00
(54)
(5)
(9)
(6)
(5)
(4)
(11)
L.E.
(9)
(12)
(7)
(9)
(11)
(6)
(3)
(10)
L.E.
(9)
(4)
(6)
AE2 WT
R926K
E951Q
D970A/K971S
E981L
K982Y
K1018P
R1056A
K1069T
D1075A
K1076A
P1077A
K1078Q
D1104P
R1107S
E1133D
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K1153R
R1155K
R1202L
c
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AE2 WT
R789E
R1056A
R1134C

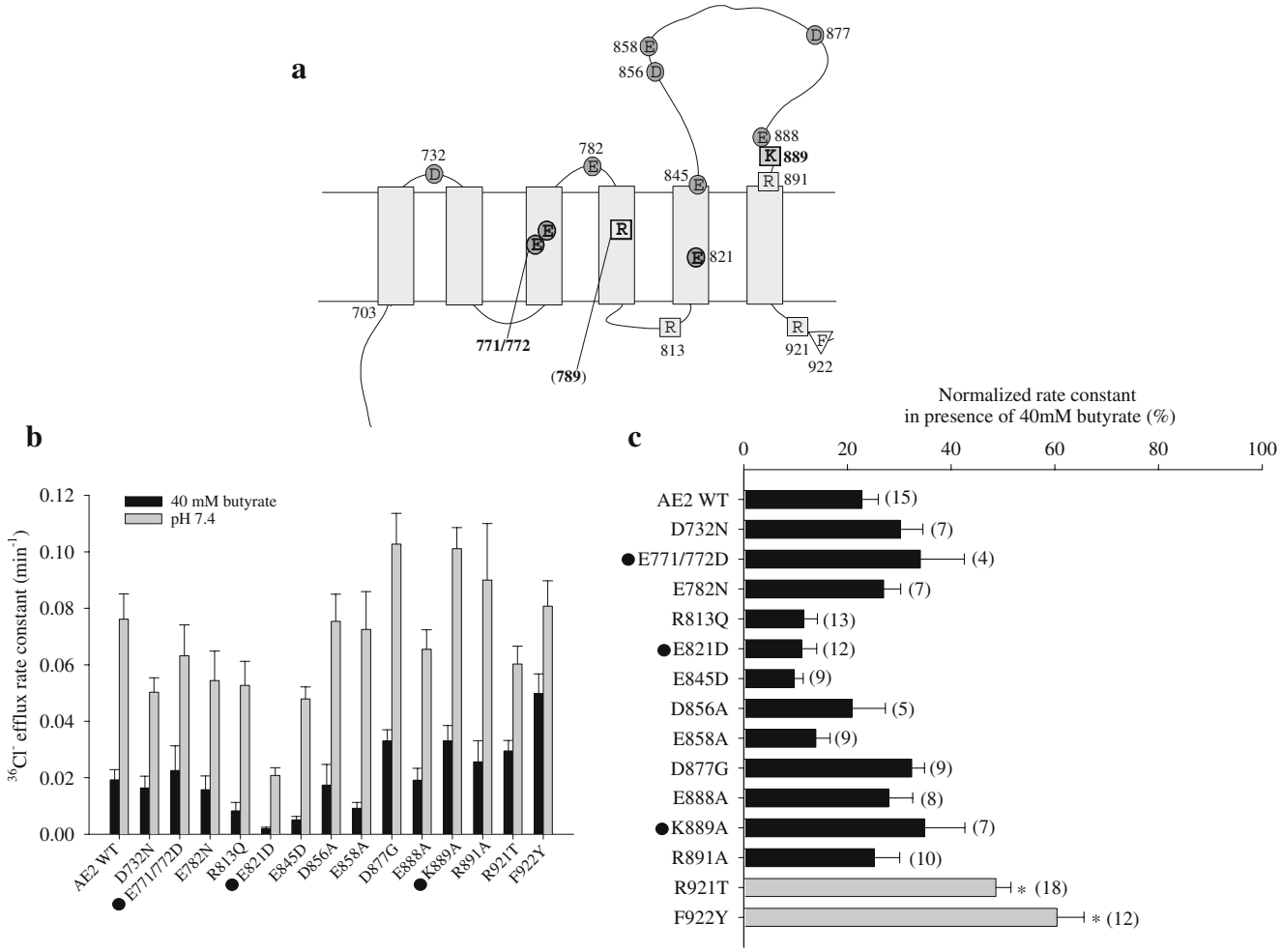


**Fig. 3** Mutations in the N-terminal half of the AE2 transmembrane domain (TM 1–6) identify residues involved in regulation by $pH_i$. **a** Position of TMD mutants within AE2 aa 703–922. Acidic residues are in *dark gray circles*; basic residues are in *light gray squares*; the single uncharged residue is in a *white inverted triangles*. Residues in *bold* are conserved in mAE1. **b** $^{36}Cl^-$ efflux rate constants for indicated TMD 1–6 mutants, in the presence (*black bars*) and subsequent absence of bath butyrate (*gray bars*), reflecting regulation by changing $pH_i$ at constant $pH_o$. **c** Normalized rate constants (±S.E.) of $^{36}Cl^-$ efflux in the presence of bath butyrate for (*n*) oocytes expressing wt AE2 or the indicated TMD mutants; *Asterisk* and *gray bars* indicate $p<0.05$ compared with wt AE2. Data from **b**. *Black circles* indicates residues that are conserved in mAE1

Fig. 3c, all active AE2 TMD mutants retained at least a degree of $pH_i$ sensitivity. The high degree of conservation throughout the SLC4 family of AE2 residues E771, E772, E821, and (to a lesser extent) K889 prompted us also to test their role in $pH_i$ regulation of AE2. The conserved charge double mutant E771D/E772D and the mutants E821D and K889A each retained $pH_i$ sensitivity. However, the AE2 TMD nonconserved charge mutants R921T and F922Y exhibited decreased $pH_i$ sensitivity compared to wild-type AE2 ($p<0.002$, Supplemental Table 1). As shown in Fig. 3a, the proposed location of these residues is the cytoplasmic loop between TM 6 and TM 7 of the AE2 TMD.

As mutagenesis of AE2 TMD His residues H1144 and H1145 shifted the $pH_o$ dependence of AE2 but did not abolish it [35], we hypothesized that nonconserved charged residues within putative TM 1–6 might also contribute to the determination of AE2 $pH_o$ sensitivity. Figure 4a compares normalized $^{36}Cl^-$ efflux activity as a function of $pH_o$ for wt AE2 and the AE2 mutants E888A and K889A. Whereas the $pH_{o(50)}$ value for E888A (6.38±0.12, $n=23$) was significantly acid-shifted compared to wt AE2 (6.83±0.04, $n=22$), the $pH_{o(50)}$ of the adjacent mutant K889A (7.21±0.13, $n=19$) was shifted to a more alkaline value (Fig. 4b; $p<0.05$). These adjacent residues, located at the C-terminal end of AE2's third extracellular loop near TM6 (Fig. 3a), were notable for their wild-type $pH_i$ sensitivity (Fig. 3c). No other AE2 mutants tested in this group were altered in $pH_o$ sensitivity, including the mutants of cytoplasmically disposed residues R921 and F922, which showed decreased sensitivity to $pH_i$, and mutants of the highly conserved residue pair E771/E772.

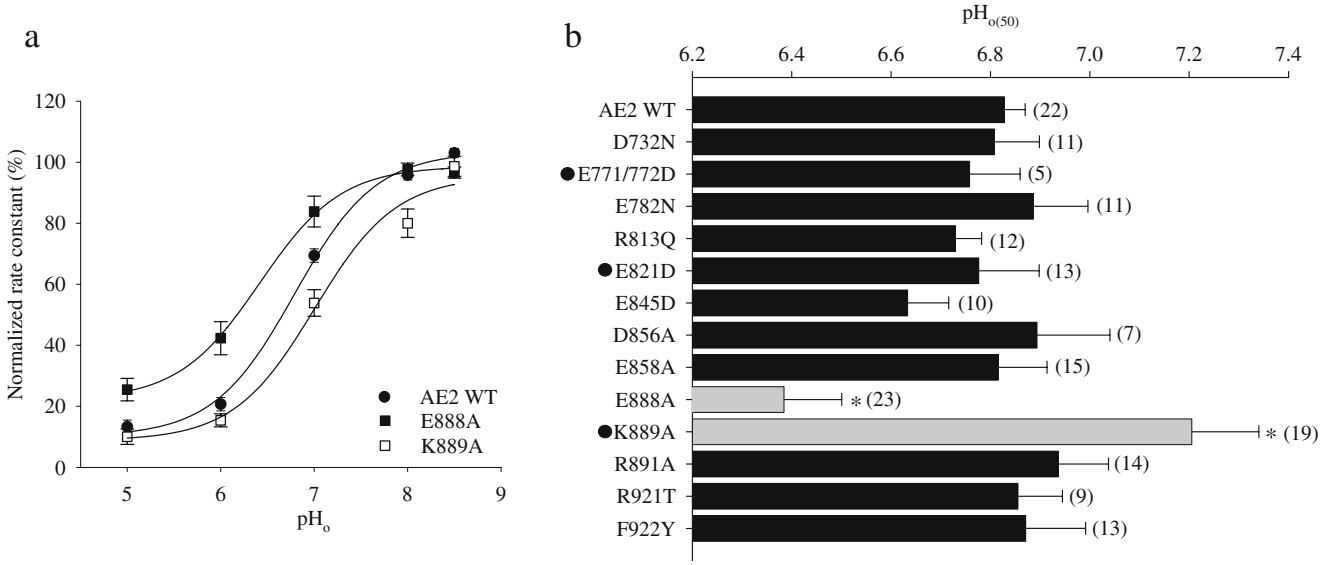


**Fig. 4** Mutations in the N-terminal half of the AE2 transmembrane domain (TM 1–6) identify residues involved in regulation by $pH_o$. **a** Regulation by $pH_o$ of normalized $^{36}Cl^-$ efflux from oocytes expressing wt AE2 ($n$=22; *filled circles*), AE2 K889A ($n$=19; *open squares*), or AE2 E888A ($n$=23; *black squares*). Values are means±S.E. **b** $pH_{o(50)}$ values for wt AE2 and the indicated TMD mutants (mean±S.E.). *Asterisk* and *gray bars* indicate mutants with $pH_{o(50)}$ values significantly different from wt AE2 ($p$<0.05). *Black circles* indicate residues that are conserved in mAE1

## AE2 residues of the C-terminal TMD (aa 926–1202) that are not conserved in AE1 contribute to the regulation of AE2 activity by pH

Figure 5a presents putative locations of nonconserved charged residues in the AE2 TMD C-terminal portion (aa 923–1202). Most of these residues are predicted to reside in the extracellular and intracellular loops and within the two putative reentrant loops. Figure 5b shows the rate constant data for $pH_i$-sensitive $^{36}Cl^-$ efflux for wild-type AE2 and for the active AE2 mutants portrayed in Fig. 5a. All active mutants retained $pH_i$ sensitivity of $^{36}Cl^-$ transport. Two AE2 mutants, P1077A located within the first proposed reentrant loop and R1107S in the adjacent putative cytoplasmic loop, displayed small but significant reductions in $pH_i$ sensitivity (Fig. 5c; $p$<0.02). In contrast, substitution of AE2 nonconserved residues D970/K971, K1153, R1155, and R1202 with their corresponding AE1 residues increases sensitivity to inhibition by acidic $pH_i$ (Fig. 5c), as we previously observed for AE2 TMD His mutant H1145A [35].

Next, we examined the consequences of these mutations in the C-terminal portion of the AE2 TMD to $pH_o$ sensitivity of $^{36}Cl^-$ efflux. Figure 6a shows that AE2 mutant D1075A (open triangles), exhibited a significantly acid-shifted $pH_{o(50)}$ value of 6.44±0.10 ($n$=19, $p$<0.05) compared to wt AE2 (6.83±0.04, $n$=22; filled circles; Supplemental Table 1). Analysis of the AE2 substitution mutants of the other 17 nonconserved charged TMD residues revealed that only mutants E981L and K982Y exhibited a similar, moderate acid-shift in $pH_o$ dependence (Fig. 6b). These three charged residue mutants with altered $pH_o$ sensitivity each exhibited wild-type $pH_i$ sensitivity (Fig. 5c). AE2 residues E981 and K982 are predicted to reside in the extracellular loop adjacent to putative TM8. AE2 residue D1075 is located in the putative first reentrant loop (Fig. 5a) at a position that in the corresponding Cys-less AE1 mutant A751C was highly accessible to chemical modification from the extracellular fluid [15].

## Discussion

AE2-mediated anion exchange is distinguished from that of its close homolog AE1 by its independent sensitivities to changing $pH_i$ and $pH_o$ across the physiological range. The roles of individual amino acid residues of the AE2 TMD in this phenotypic difference remain little understood. The present work has examined the impact on acute AE2 regulation by pH of individual mutagenic substitution of two types of charged residues of the AE2 TMD. One group of charged residues is nonconserved in the corresponding TMD positions of the relatively pH-insensitive AE1 polypeptide. A second group of charged amino acid residues was selected based on its conservation in AE1 and AE3 and, in some cases, more widely among SLC4 anion transporters. We also investigated the consequences of mutagenic substitution of several nonconserved uncharged amino acid residues of the AE2 TMD.

Individual mutagenesis of conserved charged amino acids of the AE2 TMD revealed that mutation of Arg residues R789, R1056, and R1134 led to the loss of AE2

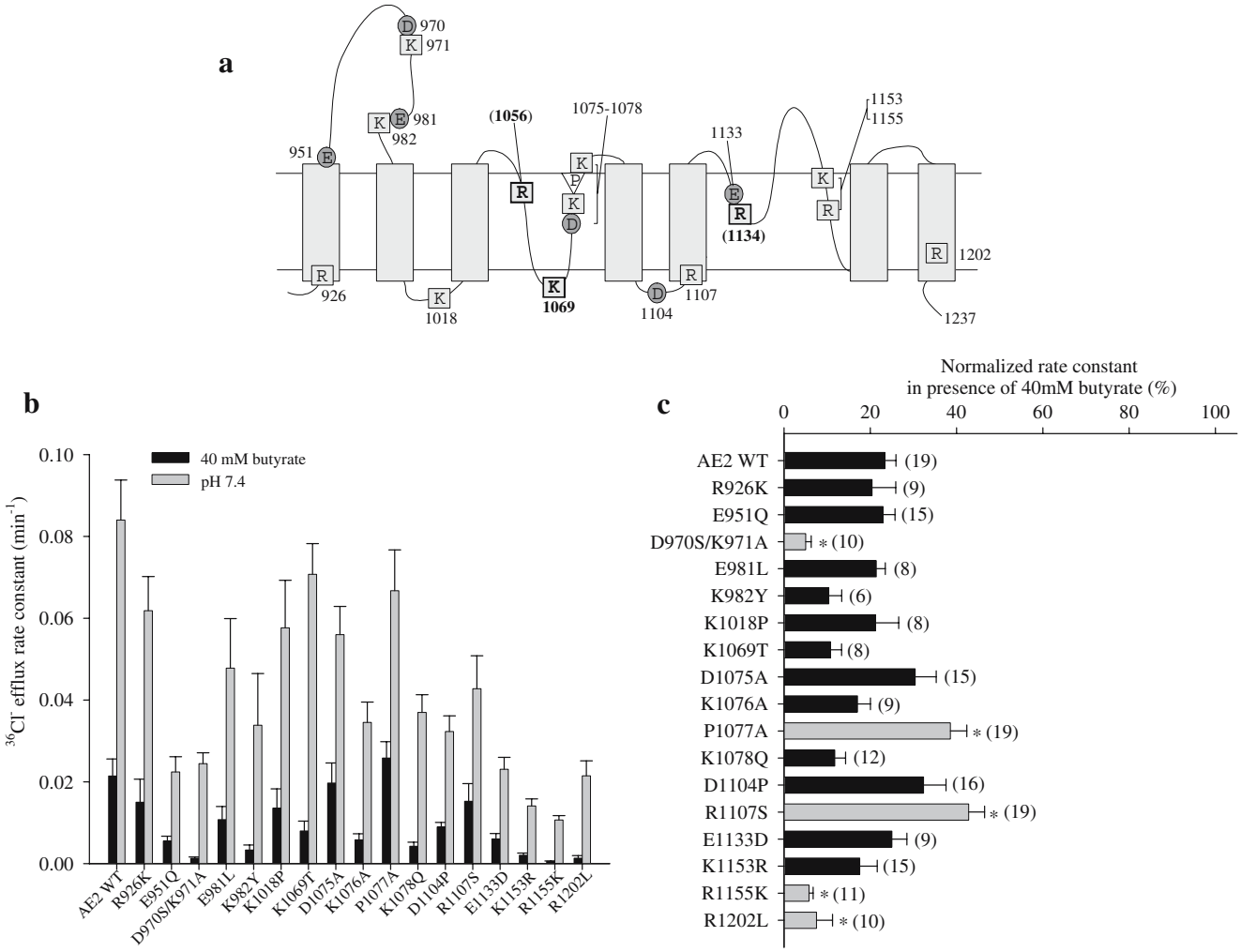


**Fig. 5** Mutations in the C-terminal half of the AE2 transmembrane domain (aa 926–1202) identify residues involved in regulation by $pH_i$. **a** Position of TMD mutants within AE2 aa 926–1202. Acidic residues are in *dark gray circles*; basic residues are in *light gray squares*; the single uncharged residue is in a *white inverted triangle*. Residues in *bold* are conserved in mAE1. **b** $^{36}Cl^-$ efflux rate constants for indicated TMD mutants, in the presence (*black bars*) and subsequent absence of bath butyrate (*gray bars*) reflecting regulation by changing $pH_i$ at constant $pH_o$. **c** Normalized rate constants (±S.E.) of $^{36}Cl^-$ efflux in the presence of bath butyrate for (*n*) oocytes expressing wt AE2 or the indicated TMD mutants; *Asterisk* and *gray bars* indicate $p<0.05$ compared with wt AE2. *Black circles* indicate residues that are conserved in mAE1

function in *Xenopus* oocytes. Several nonconserved charged residues were found to contribute to AE2 regulation by $pH_i$ (R921 and R1107) and by $pH_o$ (E888, K889, E981, K982, and D1075). Among nonconserved uncharged residues, mutation of F922 and of P1077A selectively decreased $pH_i$ sensitivity. These results suggest that the AE2 transmembrane "pH sensor" is a structure comprising multiple TMD residues differentially contributing to the sensing of $pH_i$ and $pH_o$ (Fig. 7).

## AE2 TMD residues important for $Cl^-$ transport

AE2 mutants of all nonconserved charged residues of the TMD retained $Cl^-$ transport function activity in *Xenopus* oocytes. In contrast, mutation of any one of the three conserved charged residues tested (R789, R1056, and R1134) led to loss of function, suggesting that these residues are essential to normal polypeptide stability, trafficking, and/or transport function. Intracellular-facing arginine residues have been reported to regulate anion conductance in the CFTR chloride channel [5].

Mouse AE2 R789 corresponds to hNBCe1/SLC4A4 R510 which, when mutated to His in autosomal recessive proximal renal tubular acidosis, is associated with severely decreased transport activity in *Xenopus* oocytes [19] and lack of surface expression in polarized MDCK cells [25]. A similar loss of function in oocytes was reported for mutation of the corresponding R509 of mouse AE1 to Lys, Thr, or Cys [21]. AE2 mutant R789E is expressed at or

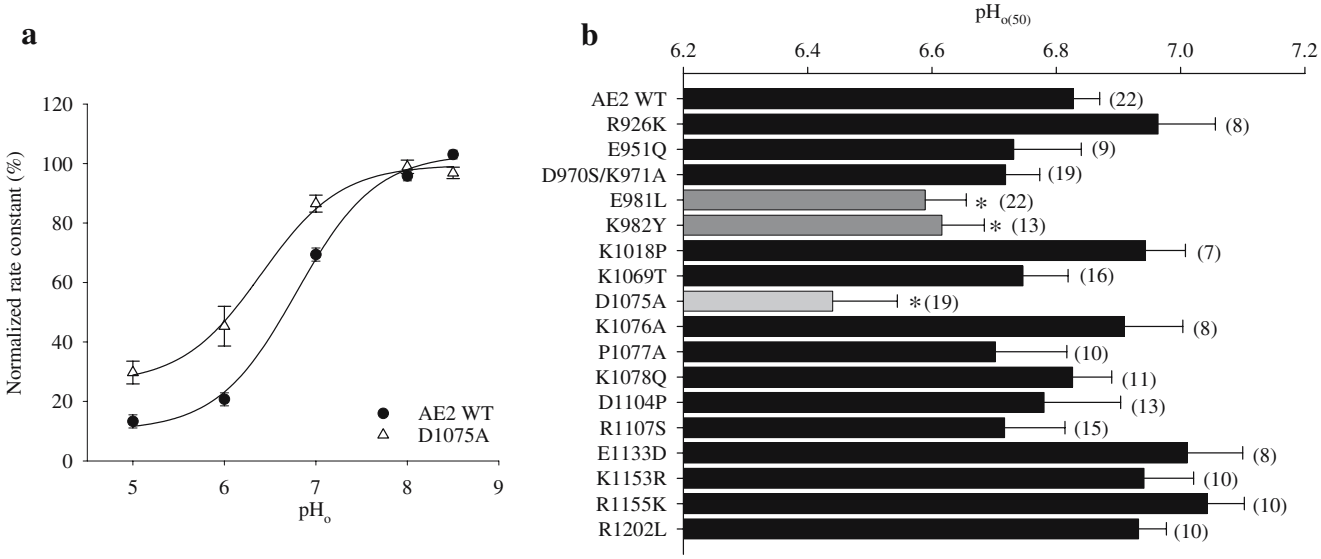


**Fig. 6** Mutations in the C-terminal half of the AE2 transmembrane domain (aa 926–1202) identify residues involved in regulation by $pH_o$. **a** Regulation by $pH_o$ of normalized $^{36}Cl^-$ efflux from oocytes expressing wt AE2 ($n$=22; *filled circles*), or AE2 D1075A ($n$=19; *open triangles*). Values are means±S.E. **b** $pH_{o(50)}$ values for wt AE2 and the indicated TMD mutants (mean±S.E.). *Asterisk* and *gray bars* indicate TMD mutants with $pH_{o(50)}$ values significantly different from wt AE2 ($p<0.05$ by Dunnett's $T$ test [*light gray*] or Student's unpaired $t$ test [*dark gray*])

near the oocyte surface (Fig. 2c), suggesting that R789 or its adjacent region of TM4 likely contributes to the stabilization of the anion translocation pathway or to the alternating access translocation mechanism.

Apparent surface expression of the mouse AE2 loss-of-function mutant R1056A (Fig. 2c) also predicts a contribution to anion translocation of this residue, located in the putative first reentrant loop (Fig. 1b). Mouse AE2 R1056 corresponds to mouse AE1 R748 which, when mutated to Lys or Gln, resulted in similar lack of transport activity [21]. The reduced transport exhibited by AE2 R1056 resembles the low transport displayed by an AE1 mutant in a residue, which by alignment is immediately adjacent. AE2 R1056 is adjacent to residue S1057, corresponding to human AE1 S731. The hAE1 mutant S731P is associated with the cryohydrocytosis form of autosomal dominant spherocytosis with cation leak, a phenotype accompanied in *Xenopus* oocytes by greatly reduced anion transport without reduction in surface expression [7].

The AE2 loss-of-function mutant R1134C in its lack of apparent surface expression (Fig. 2c) corresponds to hAE1 R808 which, when mutated to Cys in the background of the Cys-less human AE1 TMD, fails to reach the surface of HEK 293 cells [40]. AE2 R1134 also corresponds to human NBCe1 R881, the site of the R881C mutation of autosomal recessive proximal renal tubular acidosis. This NBCe1 mutant exhibits parallel ~60% reductions in wild-type transport activity and surface expression in *Xenopus* oocytes but in polarized MDCK cells fails to accumulate at the basolateral membrane [19, 36]. Similar or smaller

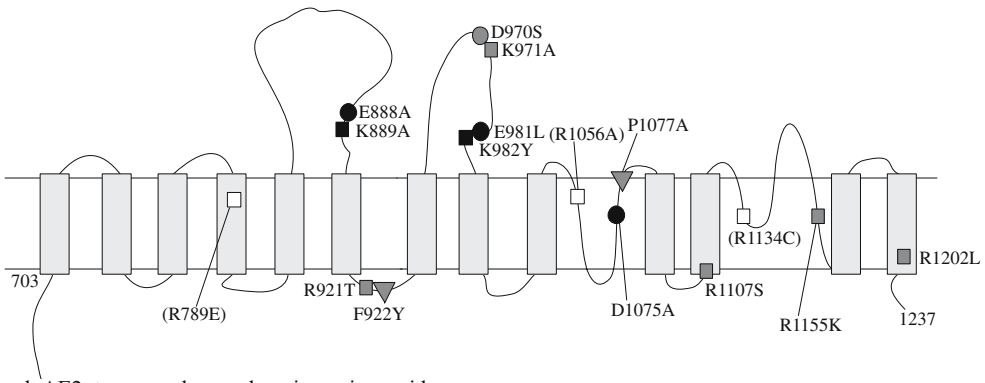


**Fig. 7** Nonconserved AE2 transmembrane domain amino acids are involved in pH regulation. Putative locations in the AE2 TMD of nonconserved amino acid residues which, when mutated, alter regulation by $pH_i$ or $pH_o$. The conserved residue K889 is also indicated. Residues involved in $pH_o$ regulation are colored *black*, and residues involved in $pH_i$ regulation are *gray*. *White squares* are "low expression" (L.E.) mutants with functional activity too low to study

proportionate reductions in anion currents have been reported to result from neutralizing mutations of Arg residues of the inner and outer vestibules of the cystic fibrosis transmembrane regulator [5, 26].

The finding of the lack of surface expression of an NBCe1 mutant in a mammalian cell line vs apparent surface expression in *Xenopus* oocytes highlights the fact that the oocyte expression system does not necessarily reflect membrane delivery of mutant polypeptide in mammalian cells. Therefore, the surface expression of AE2 mutants R1056A and R789E should be tested in a mammalian cell line to confirm the present data. These results together show the importance of conserved charged residues of the AE2 TMD to normal transport function of polypeptide at the oocyte surface.

## Nonconserved charge residues of the AE2 TMD are involved in $pH_i$ regulation

Intracellular pH sensitivity of the $K^+$ channel, ROMK, can be shifted by mutation of individual amino acid residues with a range of side chain polarities, including Lys [14, 31], Thr [10], His [8], Ile, and Leu [12]. However, none of these individual mutations completely abolished $pH_i$ regulation of ROMK. Extracellular proton inhibition of the K2P family $K^+$ channel, TASK2, was modestly attenuated by individual mutation of extracellular loop charged residues, but abolition of $pH_o$ sensitivity required concomitant mutation of four Lys and one Glu [27]. Whereas mechanistic understanding of pH regulation of ion channels is substantial, less is known about pH regulation of transporters. A recent study of the C-terminal TMD of hNBCe1 identified multiple charged residues required for *basal* transport activity [1] but did not address the consequences of these mutations to transporter regulation. Studies on the $Na^+/H^+$ exchanger, NHE1, have highlighted defined Arg and Gly residues as important elements of the TMD pH sensor of NHE1 [37].

The present work has identified multiple AE2 residues involved in AE2 regulation by $pH_i$ that are not conserved in the corresponding position of the relatively pH-insensitive AE1. These nonconserved amino acids found to be important for AE2 regulation by $pH_i$ are also conserved in most of the AE2 sequences from other species, including human, guinea pig, rat, rabbit, and chicken, suggesting a significant role in determining AE2 $pH_i$ sensitivity.

The intracellular loop proposed to connect TM6 and TM7 of AE2 differs from the AE1 sequence in only two residues, R921 and F922. Conversion of these residues to their AE1 counterparts significantly reduced AE2 inhibition by acidic $pH_i$. Individual substitution of AE1 residues into AE2 P1077 and R1107, modeled respectively to the reentrant loop between TMs 9–10 and to the cytoplasmic end of TM 11 (Fig. 1), each also produced decreased inhibition by acidic $pH_i$. The putative intracellular location of AE2 R1107 is appropriate for $pH_i$ sensing, but direct protonation would require a large local shift in guanidinium $pK_a$ by the local environment, suggesting an additional contribution of altered local electrostatics and/or conformation. The putative extracellular or reentrant location of P1077 (Fig. 1) also suggests an indirect conformational effect of mutation. Such a conformational change may lead to altered $Cl^-$ binding or release characteristics or altered rates of $Cl^-$ translocation across the AE2 permeability barrier within AE2.

## Nonconserved charge residues of the AE2 TMD are involved in $pH_o$ regulation

Exposure to diethylpyrocarbonate (DEPC) can acid-shift the $pH_o$ dependence of AE2, but mutation of individual and multiple TMD His residues of AE2 shifted $pH_o$ dependence in the *alkaline* direction [35] leading to the proposal that DEPC additionally modified non-His TMD residues.

Important roles of non-His residues in pH regulation of channels and transporters are well established. A conserved glutamate in ClC chloride/$H^+$ antiporters is important for coupling $H^+$ to the oppositely directed movement of $Cl^-$ [2, 28, 30] and in ClC $Cl^-$ channels for pH-regulated gating. Regulation of acid-sensitive cation channels by $pH_o$ requires noncontiguous regions in the extracellular domain [11]. Carboxylate residues implicated in proton gating of the voltage-gated sodium channel [6] may be shielded by a Lys residue adjacent to the pore-selectivity filter [22]. The present study presents evidence for a contribution of AE2 D1075, one of the four contiguous nonconserved amino acids DKPK (Fig. 1), to $pH_o$ regulation of AE2. As mutation of D1075 only moderately acid-shifts the $pH_{o(50)}$ value of AE2, other TMD residues are likely also involved. The $pK_a$ of the D1075 carboxylate anion (~4.8 in solution) may be alkaline-shifted by the adjacent residues K1076 and/or K1078. Acid shifts in $pH_{o(50)}$ value were observed for AE2 mutants in which residues E981 and K982 were substituted with their AE1 counterpart residues (Fig. 6b). These three AE2 residues (E981, K982, D1075) are also conserved in AE2 sequences from other species (e.g., human, rat, guinea pig), suggesting an important role in AE2 regulation by $pH_o$. The data further suggest evaluation of the combined role of these three residues using a multiple mutagenesis approach. Modest individual mutant effects on AE2 regulation by pH might, in combination, produce additive effects, as observed for pH regulation of ROMK [12] and TASK2 [27]. Interestingly, we have found that the AE2 extracellular loop (EC3) residues E888 and K889 (respectively, nonconserved and conserved in AE1) produce opposing effects on $pH_o$ dependence of AE2

(Fig. 4b). However, substitution of the entire (nonglycosylated) EC3 loop of AE1 for the native glycosylated loop of AE2 had no effect on AE2 $pH_o$ dependence (data not shown). This finding highlights the caution with which single site mutagenesis must be interpreted in the context of an intact, polytopic, transmembrane polypeptide.

Taken together, these results identify conserved charged residues that are necessary for the maintenance of basal AE2 transport and highlight nonconserved residues critical for wild-type regulation by $pH_i$ and $pH_o$ (Fig. 7). No single mutation of a nonconserved TMD residue of AE2 completely abolished regulation by pH. The current lack of a 3-D crystal structure of the AE2 TMD limits our ability to predict intramolecular interactions among TMD residues. However, unlike our results with mutations of individual TMD His residues [35], the proposed topographical location (Fig. 1) of the mutant charged and uncharged AE2 residues studied here in nearly all cases correlates well with sidedness of pH regulation in the current study. Residues, which when mutated altered inhibition by acidic $pH_i$, are predicted to be accessible to the cytoplasm. Conversely, residues, which when mutated altered regulation by $pH_o$, are predicted to reside in exofacial locations. The notable exceptions are residues D970/K971, which, although putatively located in the fourth extracellular loop of the AE2 TMD, increase sensitivity to $pH_i$ when mutated. Our data highlight the complexity of the molecular mechanisms by which pH regulates bicarbonate transporters, and suggest that multiple noncontiguous TMD amino acid residues are involved in regulation of AE2 by pH.

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