Alkaline-shifted pH Sensitivity of AE2c1-mediated Anion Exchange Reveals Novel Regulatory Determinants in the AE2 N-terminal Cytoplasmic Domain*

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The mouse anion exchanger AE2/SLC4A2 Cl⁻/HCO₃⁻ exchanger is essential to post-weaning life. AE2 polypeptides regulate pH, chloride concentration, cell volume, and transepithelial ion transport in many tissues. Although the AE2a isoform has been extensively studied, the function and regulation of the other AE2 N-terminal variant mRNAs of mouse (AE2b1, AE2b2, AE2c1, and AE2c2) have not been examined. We now present an extended analysis of AE2 variant mRNA tissue distribution and function. We show in Xenopus oocytes that all AE2 variant polypeptides except AE2c2 mediated Cl⁻ transport are subject to inhibition by acidic pH, and to activation by hypertonicity and NH₄⁺. However, AE2c1 differs from AE2a, AE2b1, and AE2b2 in its alkaline-shifted pH₄₅₀ (7.70 ± 0.11 versus 6.80 ± 0.05), suggesting the presence of a novel AE2a pH-sensitive regulatory site between amino acids 99 and 198. Initial N-terminal deletion mutagenesis restricted this site to the region between amino acids 120 and 150. Further analysis identified AE2a residues 127–129, 130–134, and 145–149 as jointly responsible for the difference in pH₄₅₀ between AE2c1 and the longer AE2a, AE2b1, and AE2b2 polypeptides. Thus, AE2c1 exhibits a unique pH₄ sensitivity among the murine AE2 variant polypeptides, in addition to a unique tissue distribution. Physiological coexpression of AE2c1 with other AE2 variant polypeptides in the same cell should extend the range over which changing pH₄ can regulate AE2 transport activity.

Na⁺-independent anion exchangers play a crucial role in the maintenance of intracellular and extracellular pH, chloride concentration, and pH₄ sensitivity. We also define the new sites within the N-terminal cytoplasmic region of AE2 whose expression pattern of AE2 variant transcripts in an extended range of mouse tissues.

MATERIALS AND METHODS

Construction of Mouse AE2 (mAE2) Variant cDNAs—Plasmid pΔX (19) was used as AE2a cDNA. cDNAs encoding alternate AE2 N-terminal variants were amplified by reverse transcription (RT)-PCR from total RNA preparations of stomach (AE2b1 and AE2b2 in 30 cycles and AE2c1 in 35 cycles) using the Expand high fidelity PCR system (Roche Diagnostics) and a manual hot start procedure (8). The forward primer for AE2b2 was 5′-TCCCCCTTCT-TCTAGGTTACC-3′ (nucleotides (nt) 31 to -10) (6). Other forward primers and a common reverse primer from exon 8 were described previously (8), yielding the following PCR products: AE2a, 1022 bp; AE2b1, 974 bp; AE2b2, 1008 bp; AE2c1, 482 bp, and AE2c2, 768 bp. PCR fragments were purified from 1% agarose gels with a QIAquick gel...
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extraction kit (Qiagen Inc.) and cloned into the “T-vector” pCR II (Invitrogen). Integrity of the cloned PCR amplification products was verified by sequencing. All mouse AE2 variant 5′-fragments were excised as EcoRI (vector-derived)-AvrII fragments and reconstructed with the remaining AE2 coding sequence in the oocyte expression vector pXT7 (20).

Construction of Mutant Mouse AE2 cDNAs—cDNAs encoding mAE2a N-terminal truncation mutants were generated by the methods described previously (12, 13) with modifications. All N-terminal truncation mutants were constructed with a 10-nt native AE2a Kozak sequence preceding the initiator Met. The hexa-Ala substitution mutant (Ala6)124–129, the triple-Ala substitution mutant (Ala3)124–126, the triple mutant P143A/P145A/P149A, and the AE2b1 mutant E121V (numbering as in AE2a) were constructed by a four-primer method (12, 13, 21). Integrity of all PCR fragments and ligation sites was confirmed by DNA sequencing of both strands.4

Tissue-specific Expression of AE2 Variant mRNA Transcripts as Detected by RT-PCR—Total RNA from freshly resected mouse stomach, duodenum, ileum, colon, choroid plexus, heart, lung, uterus, and embryonic stem cells was prepared using an RNeasy kit (Qiagen Inc.). A mouse tissue total RNA panel for brain, embryo, kidney, ovary, stomach or kidney using a PRAIII kit (Ambion, Inc.) with this modified

Measurement of Cl−/HCO3− Exchange in Oocytes, Human Embryonic Kidney (HEK) 293 Cells, and EcR-293 Cells—Oocytes previously injected with H2O or cRNA were incubated for 30 min with 5 μM bis(carboxyethyl)carboxyfluorescein (BCECF) acetoxymethyl ester at room temperature, rinsed, and mounted in a 0.8-ml superfusion chamber on a microscope stage. Cl−/HCO3− exchange was assessed by BCECF fluorescence ratio imaging of oocyte pH, changes during removal and restoration of superfusate [Cl−] (72 mM) in the presence of 5% CO2 and 24 mM HCO3−, with gluconate as the substituting anion (22). Data were acquired and analyzed with MetaFluor software (Universal Imaging, Chester, PA). dpH/dt was measured from least-squares linear fits of initial slopes.

EcR-293 cells stably expressing AE2b1 and AE2c1 (23) were grown on coverslips, incubated overnight in the absence or presence of the inducer ponasterone (5 μM), loaded for 30 min with 5 μM BCECF ace-
toxymethyl ester, and mounted on a microscope stage. Cl⁻/HCO₃⁻ exchange was measured by BCECF fluorescence ratio imaging of 30–70 subconfluent cells imaged in single ×20 visual fields during Cl⁻ removal and restoration in the presence of 5% CO₂ and 24 mM HCO₃⁻, with gluconate as the substituting anion.

The AE2b2 and AE2c1 cDNAs subcloned into pcDNA3 were transiently transfected into HEK-293 cells on glass coverslips using Lipofectamine 2000 (Invitrogen). After 48 h, the transfected cells on coverslips were incubated for 30 min with 5 μM BCECF acetoxymethyl ester and then mounted on a microscope stage. Cl⁻/HCO₃⁻ exchange was measured as described above in single visual fields containing 60–90 cells. (AE2b2 was studied in transient transfectants because our stable EcR-293 cell lines had been made prior to publication of the AE2b2 sequence (6).)

Immunodetection of mAE2 Variant Polypeptides—Groups of 10 oocytes were solubilized at 4 °C in 1% Triton oocyte lysis buffer (10 μl/oocyte) containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and Complete® protease inhibitor (Roche Diagnostics). After 30 min of intermittent shaking, the extract was centrifuged for 10 min at 4 °C in a microcentrifuge. Clarified lysate was fractionated by SDS-8% PAGE, and proteins were transferred to nitrocellulose. After incubation with affinity-purified rabbit polyclonal antibody to the mAE2 C-terminal amino acids (aa) 1224–1237 shared by all AE2 variant polypeptides (8, 24), immobilized AE2 polypeptide was visualized on Eastman Kodak X-Omat film by enhanced chemiluminescence (PerkinElmer Life Sciences).

Rabbit polyclonal antibody to mAE2b2 aa 1–11 was raised against the high pressure liquid chromatography-purified, N-terminally acetylated, C-terminally amidated peptide MDFLLRPQPEP(C). The immunizing antigen was peptide-coupled through its added C-terminal amidated Cys residue to keyhole limpet hemocyanin via 3-maleimidobenzoic acid N-hydroxysuccinimide methyl ester. Immune serum was affinity-purified over a 4-(N-maleimidomethyl)cyclohexanecarboxylic acid N-hydroxysuccinimide methyl ester-coupled peptide antigen column. HEK-293 cells transiently transfected with AE2b2 and AE2c1 cDNAs as described above were fixed in 2% paraformaldehyde and immunostained with the affinity-purified anti-AE2 antibodies in the presence of 12 μg/ml peptide antigen or irrelevant peptide as described previously (8, 23, 24). Images were acquired with a Bio-Rad MRC1024 laser scanning confocal microscope.

RESULTS

Tissue Distribution of mAE2 Variant mRNA Expression—The five mAE2 variant transcripts encode the five predicted AE2 polypeptides schematized in Fig. 1. We examined the tissue distribution of the transcripts encoding these five polypeptides. AE2a mRNA was detected in all 17 tissues screened by RT-PCR (Fig. 2, A–D). AE2b1 and AE2b2 were also detected in all tissues analyzed, with the single exception of spleen. Stomach mRNA levels of AE2b1 appeared to be approximately equivalent to those of AE2a. Colonic levels of AE2b1 appeared to be slightly less abundant than those of AE2a. The apparent relative expression levels of AE2a, AEb1, and AE2b2 in mouse kidney (Fig. 2A) paralleled

![FIGURE 1. Schematic of the five polypeptide products of mAE2 transcripts. Variant N-terminal amino acids sequences are shown. The AE2c1 and AE2c2 diagrams show the first shared amino acids (AE2a numbering). Cyto, N-terminal cytoplasmic domain (705 aa long in AE2a); TMD, transmembrane domain.](image)

![FIGURE 2. Tissue distribution of AE2 N-terminal variant mRNAs in mouse tissues. A–D, total RNA from the indicated tissues was tested by RT-PCR for the presence of all known AE2 variant transcripts. The cycle number is listed at bottom of each lane. Specific RT-PCR product lengths are as follows: AE2a, 1022 nt; AE2b1, 974 nt; AE2b2, 1008 nt; AE2c1, 482 nt; and AE2c2, 768 nt. Uniformity of the RT reaction was confirmed for each tissue sample by RT-PCR of β-actin (23 cycles, not shown). E, RPA was performed with the indicated micrograms of total RNA from mouse stomach and kidney. Note that the level of AE2c mRNA (AE2c1 + AE2c2) relative to that of AE2a + AE2b is higher than apparent from band intensities since the signal strength of the randomly labeled probe must be evaluated according to the length of the protected fragments. ES cells, embryonic stem cells.](image)
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those detected by RT-PCR in rat kidney, in which most of the AE2b1 was from the inner stripe of the outer medulla. 6

Stomach was the only tissue studied that expressed substantial levels of AE2c1 mRNA. Trace AE2c1 expression was detected in liver, colon, choroid plexus, and uterus after 38 cycles of PCR with overexposure of gels (data not shown). AE2c2 expression was more widespread, with PCR products detected in all tissues examined except spleen, duodenum, brain, heart, and testis. 7 The AE2c2 mRNA abundance relative to AE2c1 in stomach (Fig. 2C) is likely an underestimate of the real value, as noted in control amplifications with this single AE2c primer pair (see "Materials and Methods").

The ability to detect AE2c transcripts by RPA was tested in mouse stomach and kidney (Fig. 2E). The structures of the AE2 variant transcripts do not permit design of a single RPA probe capable of detecting all five individual transcripts. Thus, the RPA probe was chosen to discriminate a single upper band representing combined AE2a, AE2b1, and AE2b2 and a single lower band of combined AE2c1 and AE2c2. The proportional signals detected in 10, 3, and 1 μg of stomach RNA demonstrated the quantitative nature of the RPA. The ratio of gastric AE2a/AE2b1/AE2b2 to AE2c1/AE2c2 detected by RPA (Fig. 2E) seemed lower than evidenced by RT-PCR (in which the differences are exponential) (Fig. 2, A and C). This difference highlights the potential pitfalls of comparing RT-PCRs not employing identical primer pairs. However, the kidney AE2c2 transcript clearly detected by a 38-cycle RT-PCR amplification (Fig. 2A) was virtually undetectable by RPA (Fig. 2E), emphasizing the role of RT-PCR in high sensitivity transcript detection.

Four of the Five mAE2 Variant Polypeptides Are Functional in Xenopus Oocytes and in Mammalian Cells—All AE2 variant polypeptides (Fig. 1) were expressed in Xenopus oocytes previously injected with equivalent amounts of cRNA. The extensively studied AE2a polypeptide (Fig. 1) were expressed in emphasizing the role of RT-PCR in high sensitivity transcript detection. Of the most active variants, AE2b1 and AE2b2 (Fig. 3A). 36Cl− efflux assays revealed that AE2b1 and AE2b2 mediated the highest rates of Cl−/Cl− exchange (Fig. 3B). AE2a and AE2c1 exhibited lower, roughly equivalent rates of anion transport. The AE2c1 mediated no detectable 36Cl− efflux activity (Fig. 3B), as was shown earlier for AE2a (25).

We previously described immunodetection of muristerone-induced AE2b1 and AE2c1 overexpression in stably transfected EcR-293 cells (23). Fig. 4A shows immunodetection of AE2b2 in transiently transfected HEK-293 cells by antibodies to the common AE2 C-terminal peptide (upper panels) and to the AE2b2-specific N-terminal peptide (lower panels). Transient transfection of HEK-293 cells with cDNAs encoding AE2b2 or AE2c1 led to increased Cl−/HCO3− exchange activity (Fig. 4, B and C). In addition, Cl−/HCO3− exchange activity was induced by the muristerone analog ponasterone in clonal EcR-293 cell lines stably transfected with AE2b1 or AE2c1 (Fig. 4, D and E), consistent with the previously described parallel induction of heterologous AE2b1 and AE2c1 polypeptides (23).

Regulation of AE2 Variant Polypeptides by NH4+

S. M. Wall, personal communication.

7 Fig. 2 also shows, in choroid plexus and at lower abundance in kidney, jejunum, colon, lung, uterus, and thymus, an RT-PCR product of the AE2c2 primer pair migrating above the AE2c2 band (under further characterization) (B. E. Shmukler and S. L. Alper, unpublished data).

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FIGURE 4. mAE2 variants mediate Cl⁻/HCO₃⁻ exchange in mammalian cells. A, immunolocalization of the AE2b2 polypeptide in HEK-293 cells transiently transfected with AE2b2 cDNA. Fixed cells were immunostained with anti-AE2a antibody to the shared C-terminal aa 1224–1237 (upper panels) or with antibody specific for the N-terminal 11 aa of AE2b2 (lower panels) in the presence of irrelevant peptide (left panels) or of specific peptide antigen (right panels). B, Cl⁻/HCO₃⁻ exchange activity portrayed by traces of pH_i versus time, recorded during the removal and subsequent restoration of bath Cl⁻. Traces are from representative coverslips containing HEK-293 cells either mock-transfected or transfected with AE2c1 cDNA. C, summarized Cl⁻/HCO₃⁻ exchange activity results recorded from n coverslips of mock-transfected (control) HEK-293 cells or from cells transiently transfected with AE2b2 or AE2c1 cDNA (means ± S.E.). Initial resting pH values were indistinguishable. Coverslips with transiently transfected cells included some non-transfected cells within the region of interest, and so dpH_i/dt values represent underestimates of the heterologous activities of AE2b2 and AE2c1. D, Cl⁻/HCO₃⁻ exchange activity portrayed by traces of pH_i versus time, recorded during the removal and subsequent restoration of bath Cl⁻. Traces are from coverslips containing EcR-293 cells stably transfected with AE2c1 cDNA and pretreated overnight in the absence or presence of the transgene inducer ponasterone. E, summarized Cl⁻/HCO₃⁻ exchange activity results recorded from n coverslips of EcR-293 cells stably transfected with AE2b1 or AE2c1 cDNA after pretreatment without or with ponasterone (means ± S.E.).
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AE2a (n = 25). This finding was unexpected in view of the wild-type $\text{pH}_{\text{c(50)}}$ values for AE2a N-terminal deletion mutants $\Delta_{\text{N99}}$ and $\Delta_{\text{N310}}$ (12, 13). Moreover, all previous N-terminal cytoplasmic domain mutants in AE2 that altered $\text{pH}_i$ sensitivity shifted the $\text{pH}_{\text{c(50)}}$ to more acidic rather than more alkaline values (13–15).

Regulation of N-terminal Truncation Mutants by Intracellular and Extracellular pH—Because AE2a mutant $\Delta_{\text{N99}}$ exhibited a wild-type $\text{pH}_{\text{c(50)}}$ and because AE2c1 is equivalent to the nominal AE2a mutant $\Delta_{\text{N198}}$, we hypothesized that the region of AE2a between aa 99 and 198 would include residues responsible for the novel alkaline-shifted $\text{pH}_{\text{c(50)}}$ for AE2c1. AE2a N-terminal truncation mutants $\Delta_{\text{N120}}, \Delta_{\text{N150}},$ and $\Delta_{\text{N175}}$ (Fig. 7A) each exhibited sufficient $^{36}\text{Cl}^-$ efflux activity (Fig. 7B) to allow tests of $\text{pH}_i$ and $\text{pH}_o$ sensitivity. All mutants exhibited a wild-type phenotype for inhibition upon $\text{pH}_i$ acidification by bath butyrate addition and activation upon intracellular alkalinization by butyrate removal (Fig. 7, C–E). The $\text{pH}_{\text{c(50)}}$ for AE2a mutant $\Delta_{\text{N120}}$ (6.95 ± 0.07, n = 17) did not differ significantly from that for wild-type AE2a (6.80 ± 0.05, n = 25). In contrast, AE2a mutants $\Delta_{\text{N150}}$ and $\Delta_{\text{N175}}$ both demonstrated alkaline-shifted $\text{pH}_{\text{c(50)}}$ values of 7.76 ± 0.08 (n = 24) and 7.61 ± 0.09 (n = 17), respectively (Fig. 7, F–H), in comparable magnitude to the alkaline-shifted $\text{pH}_{\text{c(50)}}$ for AE2c1.

The difference in $\text{pH}_{\text{c(50)}}$ values between AE2a truncation mutants $\Delta_{\text{N120}}$ and $\Delta_{\text{N150}}$ focused attention on the region between aa 121 and 150. We therefore generated and studied the sequential AE2a deletion mutants $\Delta_{\text{N125}}, \Delta_{\text{N130}}, \Delta_{\text{N135}}, \Delta_{\text{N140}},$ and $\Delta_{\text{N145}}$ (Fig. 8A). All these deletion mutants were active (Fig. 8B), and inhibition of $\text{Cl}^-/\text{Cl}^-$ exchange by bath butyrate-induced intracellular alkalinization was similar in all deletion mutants tested (Fig. 8, C–E). However, inhibition was less pronounced than in the physiological AE2 variants (Fig. 6C) and in deletion mutants $\Delta_{\text{N120}}, \Delta_{\text{N150}},$ and $\Delta_{\text{N175}}$ (Fig. 7E) ($p < 0.05$ for all mutants except $\Delta_{\text{N130}}$). Although the $\text{pH}_{\text{c(50)}}$ values for mutants $\Delta_{\text{N125}}$ and $\Delta_{\text{N130}}$ were close to the wild-type value (6.72 ± 0.06 (n = 11) and 6.90 ± 0.06 (n = 18), respectively), mutants $\Delta_{\text{N135}}, \Delta_{\text{N140}},$ and $\Delta_{\text{N145}}$ each showed a moderate alkaline shift (respective $\text{pH}_{\text{c(50)}}$ values of 7.25 ± 0.1 (n = 17), 7.13 ± 0.09 (n = 13), and 7.17 ± 0.07 (n = 16); $p < 0.01$), although less than for mutant $\Delta_{\text{N150}}$ or AE2c1 (Fig. 8, F–H). Therefore, two small, nearby, but nonadjacent regions of the AE2a N-terminal cytoplasmic domain encompassing aa 131–135 and 145–150 appear to be responsible for the majority of the difference between the $\text{pH}_{\text{c(50)}}$ values for AE2a and AE2c1.

Roles of Acidic and Pro-rich Sequences and of a Polymorphic Variant in Regulation of AE2 $\text{pH}_i$ Sensitivity by Amino Acids 121–150—The AE2a region between aa 125 and 130 overlaps with an acidic sequence highly conserved among AE2 and AE3 polypeptides (Fig. 9A). To test the importance of this sequence to the alkaline-shifted $\text{pH}_{\text{c(50)}}$ for AE2a mutant $\Delta_{\text{N135}}$ (and to that for AE2c1), we constructed the hexa-Ala substitution mutant (Ala$_6$)$_{\text{124–129}}$ and the triple-Ala substitution mutant (Ala$_3$)$_{\text{124–126}}$. The $\text{pH}_i$ sensitivity of mutant activity was unchanged (Fig. 9, C–E). However, (Ala$_6$)$_{\text{124–129}}$ exhibited an alkaline-shifted $\text{pH}_{\text{c(50)}}$ similar to that for AE2 $\Delta_{\text{N135}}$, whereas the $\text{pH}_i$ sensitivity of (Ala$_3$)$_{\text{124–126}}$ resembled that of wild-type AE2a (Fig. 9, F–H). Thus, residues 127–129 appear to contribute to the alkaline-shifted $\text{pH}_i$ sensitivity of AE2c1 and replicate the moderate alkaline-shifted $\text{pH}_{\text{c(50)}}$ for deletion mutant $\Delta_{\text{N135}}$.

AE2a aa 145–150 overlap with a highly conserved proline-rich sequence fitting the consensus for a glycogen synthase kinase-3 phosphorylation site at either Ser$^{144}$ or Thr$^{148}$ (Fig. 9A). The importance of this region was addressed by the construction and study of the AE2a triple mutant P143A/P145A/P149A. Fig. 9 (C–E) demonstrates that $\text{pH}_i$ regulation of these mutants did not differ from that of AE2a. Ala substitution of all three Pro residues did not replicate either the moderate alkaline shift of AE2 mutant $\Delta_{\text{N135}}$ or the greater alkaline shifts of mutant $\Delta_{\text{N150}}$ and AE2c1 (Fig. 9F). These data do not support a required role for this proline-rich region in controlling AE2 $\text{pH}_i$ sensitivity.

The human AE2 polymorphism E122V corresponds to mAE2 E121V within the AE2a region of aa 121–150 implicated in regulation of $\text{pH}_i$ sensitivity. This non-conserved polymorphism replaces negatively
charged Glu with uncharged Val. The functional consequence of this substitution was tested in the background of mAE2b1. Neither pH sensitivity nor pH$_s$ sensitivity of AE2b1 was altered by the E121V substitution (Figs. 6, E and F; and 10, A–D), suggesting that this polymorphism likely does not contribute to the pH sensitivity of human AE2.

**DISCUSSION**

The widely expressed mAE2 gene is the source of at least five defined transcripts arising from alternative promoter usage. The AE2a transcript encoding the longest AE2 polypeptide (26, 27) has until now been the only functionally characterized AE2 polypeptide (12–18, 28, 29). The discovery of AE2b1, AE2c1, and AE2c2 transcripts in rat (9) was followed by detection of AE2c2 in mouse (8) and the discovery of AE2b2 and AE2c1 and exposed sequentially to baths of increasing pH$_o$ values; E, normalized $^{36}\text{Cl}^-/\text{H}^+$ efflux rate constants as a function of pH$_o$ for oocytes expressing the indicated AE2 variant polypeptides; F, pH$_s$ regulation of AE2c1 is alkaline-shifted. The pH$_{s(o)}$ values for n oocytes expressing AE2a, AE2b1, AE2b2, and AE2c1 are indicated. The data are the means ± S.E. for oocytes previously injected with 10 ng of cRNA. This lack of function did not correlate with the level of polypeptide accumulation. AE2c2 was also a functional Cl$^-$/HCO$_3^-$ exchanger in both oocytes and mammalian cells, and AE2b1 and AE2b2 mediated Cl$^-$/HCO$_3^-$ exchange in mammalian cells. AE2c2 did not exhibit detectable Cl$^-$/HCO$_3^-$ exchange function in oocytes previously injected with 10 ng of cRNA. This lack of function was not due to the absence of AE2c2 polypeptide accumulation because the abundance of AE2c2 was equivalent to that of the highly active AE2b1 and AE2b2. The oocyte surface expression of AE2c2 compared with that of the functional AE2 variant polypeptides remains to be examined. The minimal functional activity of mAE2c2 in oocytes is particularly interesting insofar as rat AE2c2 mRNA does not encode an AE2c2 N-terminally elongated polypeptide. Instead, rat AE2c2 and AE2c1 mRNAs both encode the single rat AE2c1 polypeptide (8, 9, 24).

**AE2c1 Exhibits a Unique pH$_s$ Sensitivity Due to the Absence of a Novel pH$_s$ Sensor Region**—AE2 polypeptides functional in Xenopus oocytes were also functional in mammalian cells. All functional AE2 variant polypeptides expressed in Xenopus oocytes were activated by both hypertonicity and NH$_4^+$, although AE2b2 showed a lower degree of activation by hypertonicity. All AE2 variants were inhibited by acidic pH$_o$, albeit to different degrees. However, the four functional AE2 polypeptides differed in pH$_s$ sensitivity. Although AE2b1 and AE2b2 exhibited pH$_{s(50)}$ sensitivity nor pH$_s$ sensitivity of human AE2.
values indistinguishable from that for AE2a, the pH\(_{50}\) for AE2c1 was shifted to alkaline values by almost 1 pH unit (Fig. 6). This alkaline shift in pH\(_{50}\) is unique among the shifts in pH\(_{50}\)s produced by directed mutations in the AE2 N-terminal cytoplasmic domain, all of which have acid-shifted pH\(_{50}\) values. The initiator Met of AE2c1 is aa 199 of AE2a. The pH\(_{50}\) values for AE2a mutants H9004 N99 (12) and H9004 N310 (13) were known to be indistinguishable from that for wild-type AE2a. Therefore, the AE2a sequence whose absence explains the distinct AE2c1 pH\(_{50}\) was sought between AE2a aa 100 and 198. The initial N-terminal deletion mutants studied confirmed the existence of such sequence between AE2a aa 121 and 150 (Fig. 7).

**Determinants of the Novel pH\(_{50}\) Regulatory Structure Comprise at Least Two Noncontiguous but Adjacent Sequences—**The difference in pH\(_{50}\) sensitivity between AE2c1 and other functional AE2 polypeptides was investigated more intensively by study of deletion and substitution mutants between aa 121 and 150 of the AE2 N-terminal cytoplasmic domain. Functional analysis of deletion mutants suggested at least two regions of importance, between aa 125 and 135 and between aa 145 and 150 (Fig. 8). The former region was notable for the highly conserved polyacidic stretch of AE2a aa 124–129. The H9004 N135 phenotype was replicated by substitution mutant (Ala6)124–129, but not by mutant (Ala3)124–126, confirming a contribution of residues 127–129 to AE2 regulation by pH\(_{50}\) (Fig. 9). This acidic stretch might contribute to a predicted “moderate stringency” consensus phosphorylation site for casein kinase-1 or -2 at Ser131 (available at www.scansite.mit.edu). The mAE2 E122V equivalent of the common human single-nucleotide coding polymorphism E121V was shown not to modify AE2 regulation by pH\(_{50}\) or pH\(_{i}\) (Fig. 10).

Near and within aa 145–150 are three highly conserved Pro residues that constitute part of a predicted consensus Src homology-3 domain-binding site for phospholipase-\(\gamma\) and that contribute to a predicted moderate stringency consensus phosphorylation site for glycogen synthase kinase-3 at either Ser\(^{148}\) or Thr\(^{148}\) (available at www.scansite.mit.edu). However, the AE2 triple-Ala substitution mutant P143A/P145A/
P149A did not suffice to shift pH
_\text{w}(50)_ to a more alkaline value (Fig. 9). Thus, the mechanism by which residues 145–150 contribute to regulation of AE2 pH
_\text{w}(50)_ remains to be determined. The entire difference in pH
_\text{w}(50)_ values between AE2a/AE2b1/AE2b2 and AE2c1 can be plausibly explained by the additive (but not obligatorily interactive) contributions of AE2a aa 127–135 and 146–150, perhaps with additional residues immediately adjacent.

The region of AE2a aa 121–150 has no correspondent within that portion of the AE1/SLC4A1 N-terminal cytoplasmic domain for which a 3.4-Å x-ray crystal structure is available. The secondary structure of aa 121–150 is predicted to include an \(\alpha\)-helical stretch in its middle portion, but 4 of the 14 following residues are prolines. The degree of physiological interaction of the pH
_\text{w}(50)_ regulatory residues within this region with the previously described pH
_\text{w}(50)_ regulatory residues within aa 336–347 (14) and in adjacent stretches (15) remains to be determined. Also to be determined is how regulation of pH
_\text{w}(50)_ by the novel sites identified in this work interacts with the independent inhibitory regulation by intracellular protons and the independent activating stimuli of NH\(_4^+\) and of hypertonicity, each regulated by other residues of AE2.

Implications of the Alkaline-shifted pH
_\text{w}(50)_ of AE2c1 for Other Determinants of pH
_\text{w}(50)_ Sensitivity within the Amino Acid Sequence Unique to AE2a—Previous mutational structure-function studies of mAE2 have generated many mutants that exhibit an “acid-shifted pH
_\text{w}(50)_ (50) value” for Cl\(^-\)/HCO\(_3^-\) exchange. The AE2c1 polypeptide represents the first instance of an alkaline-shifted pH
_\text{w}(50)_ value (compared with AE2a) in a physiological AE2 isoform (Fig. 6). This property was surprising in view of the unchanged pH
_\text{w}(50)_ values for the AE2a N-terminal truncation mutants \(\Delta_{N99}\) and \(\Delta_{N310}\) (12, 13). Moreover, further truncation, as well as indi-

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**FIGURE 8.** Wild-type pH
_\text{w}(50)_ sensitivity of AE2-mediated Cl\(^-\)/HCO\(_3^-\) exchange requires at least two noncontiguous residues or regions between AE2a aa 121 and 150. A, schematic of five serial N-terminal deletion mutants encompassing AE2a aa 121–150: \(\Delta_{N125}\); mean \(^35\text{Cl}\) efflux rate constants at pH\(_7.4\) in ND96 buffer for \(n\) oocytes expressing the N-terminal deletion mutants studied; B, \(^35\text{Cl}\) efflux rate constants in the presence and subsequent absence of 40 mM butyrate, followed by inhibition with DIDS (200 \(\mu\)M); C, \(^35\text{Cl}\) efflux time course during sequential pH
_\text{w}(50)_ changes for representative oocytes previously injected with H\(_2\)O or cRNA encoding the indicated wild-type or mutant AE2a polypeptide; D, normalized \(^36\text{Cl}\) efflux rate constants as a function of pH
_\text{w}(50)_ for oocytes expressing \(\Delta_{N125}\), \(\Delta_{N135}\), or \(\Delta_{N150}\) (data reproduced from Fig. 6); E, pH
_\text{w}(50)_ (50) values (means \(\pm\) S.E.) for \(n\) oocytes expressing the indicated mutant AE2a polypeptides. \(\Delta_{N150}\) data are from Fig. 6. Gray bars indicate significant difference from mAE2a (\(p < 0.05\)).
Alkaline-shifted pH Sensitivity of AE2c1

Individual missense mutations beyond aa 310, also acid-shifted the pH$_{50}$ for Cl$^-$/HCO$_3^-$ exchange (13–15). These data suggest that investigation of AE2a residues between positions 199 and 310 will uncover groups of residues whose mutation or removal will revert the alkaline-shifted pH$_{50}$ for AE2c1 back to that shared by wild-type AE2a and N310, as suggested by preliminary experiments.8 The relationship between these regions and the highly conserved aa 336–347, mutations of which acid-shift the pH$_{50}$ for AE2 (14), remains unknown. The properties of the multiple AE2 N-terminal cytoplasmic domain mutants and natural variants studied to date suggest a complex folded structure in which many mutations can alter presumed regulatory interaction with the transmembrane domain to produce opposing shifts in the pH$_{50}$ sensitivity of anion transport. Although the first half of this 705-aa AE2 N-terminal cytoplasmic domain is of unknown structure, we have previously modeled the domain’s second half (14, 15) on the pH 4.8 crystal structure of the corresponding AE1 domain (31). However, this modeling should be interpreted cautiously, as recent lanthanide resonance energy transfer data suggest a considerably more elongated native structure of the AE1 N-terminal cytoplasmic domain than indicated by the crystal structure (32).

Physiological Role of AE2c1 Expression in Stomach—The restriction of AE2c1 expression to stomach is appropriate if AE2c1 is expressed in parietal cells, where it would be exposed to high [HCO$_3^-$] in the basolateral interstitial space. In an elutriation-enriched fraction of rabbit parietal cells, AE2c transcripts are indeed present, and semiquantitative RT-PCR suggested that proportional AE2 mRNA levels are AE2b >

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8 C. E. Kurschat, A. K. Stewart, and S. L. Alper, unpublished data.
AE2c > AE2a (33). Although Fig. 2 and the results of Lecanda et al. (6) suggest that proportional AE2a expression is considerably higher in mouse stomach, the expression of AE2c1 together with AE2a, AE2b1, or AE2b2 in the basolateral membrane of parietal cells should broaden the pH \textit{versus} activity profile of Cl\(^{-}/\text{HCO}_3\) exchange and allow regulation of anion exchange activity at the prevailing alkaline values of interstitial pHi. This contribution of AE2c1 might increase the stability of parietal cell pH\(_i\) during active luminal H\(^+\) secretion beyond that achievable by AE2a, AE2b1, or AE2b2. AE2c1 expression should help sustain maximal rates of parietal cell acid secretion with regulatory activation by interstitial alkaline pHi\(_o\) countering or overcoming the adverse gradient effect of increased interstitial [HCO\(_3\)] resulting from AE2 activity. Conversely, elevation-enriched rabbit gastric mucose cells express AE2c1 at low levels (31), as might be appropriate for a HCO\(_3\) -secreting cell expected to acidify its adjacent interstitium. Thus, it will be of interest to learn about the acid secretory function of gastric mucosa in the mouse strain engineered to lack AE2a, AE2b1, and AE2b2 (11). No human genomic DNA entry in the annotated data base predicts a human AE2c1 transcript, and no reported human expressed sequence tags encode either AE2c1 or AE2c2. Moreover, the codons corresponding to mA2e Met\(^{199}\) encode Ala and Val in the human and rabbit AE2 genes, respectively. Thus, the species specificity of AE2c1 polypeptide expression may contribute to differences in regulation of gastric acidification by mouse and rat in comparison with human and rabbit.

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9. However, the gastric vascular anatomic studies of Gannon et al. (34, 35) suggest that the bicarbonate secreted basolaterally by parietal cell AE2 is carried by the periglandular capillary network to the interstitium underlying the mucosal surface cells. There, it can counter back-leak of luminal acid and any basolateral acid secretion accompanying surface cell bicarbonate secretion. Thus, the interstitium of gastric surface cells may also be alkaline.

10. For specific detection of AE2c2 transcripts, Lecanda et al. (6) used intron 5 forward primer MAE59 with exon 6 reverse primer MAE62. The cDNA amplification product of this primer pair spans no exon-exon exonsitional splice junction and so cannot discriminate between amplification products of identical length derived from cDNA and from traces of genomic DNA always containing total RNA preparations isolated from tissues. Use of this primer pair with total RNA (of unspecified DNase pretreatment status) produced a relatively abundant AE2c2 band after 35 cycles (6). In contrast, with the intron 5 forward primer and the exon 8 reverse primer used in Fig. 2, 38 cycles of amplification were required for detection of AE2c2 in all tissues except stomach and choroid plexus. The difference in reported AE2c2 mRNA abundance may also simply reflect different amplification efficiencies of the different AE2c2 primer pairs.
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