Acute regulation of mouse AE2 anion exchanger requires isoform-specific amino acid residues from most of the transmembrane domain

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The widely expressed anion exchanger polypeptide AE2/SLC4A2 is acutely inhibited by acidic intracellular (pH_i), by acidic extracellular pH (pH_o), and by the calmodulin inhibitor, calmidazolium, whereas it is acutely activated by NH_4^+. The homologous erythroid/kidney AE1/SLC4A1 polypeptide is insensitive to these regulators. Each of these AE2 regulatory responses requires the presence of AE2's C-terminal transmembrane domain (TMD). We have now measured $^{36}Cl^-$ efflux from *Xenopus* oocytes expressing bi- or tripartite AE2–AE1 chimeras to define TMD subregions in which AE2-specific sequences contribute to acute regulation. The chimeric AE polypeptides were all functional at pH_o 7.4, with the sole exception of $AE2_{(1-920)}/AE1_{(613-811)}/AE2_{(1120-1237)}$. Reciprocal exchanges of the large third extracellular loops were without effect. AE2 regulation by pH_i, pH_o and NH_4^+ was retained after substitution of C-terminal AE2 amino acids 1120–1237 (including the putative second re-entrant loop, two TM spans and the cytoplasmic tail) with the corresponding AE1 sequence. In contrast, the presence of this AE2 C-terminal sequence was both necessary and sufficient for inhibition by calmidazolium. All other tested TMD substitutions abolished AE2 pH_i sensitivity, abolished or severely attenuated sensitivity to pH_o and removed sensitivity to NH_4^+. Loss of AE2 pH_i sensitivity was not rescued by co-expression of a complementary AE2 sequence within separate full-length chimeras or AE2 subdomains. Thus, normal regulation of AE2 by pH and other ligands requires AE2-specific sequence from most regions of the AE2 TMD, with the exceptions of the third extracellular loop and a short C-terminal sequence. We conclude that the individual TMD amino acid residues previously identified as influencing acute regulation of AE2 exert that influence within a regulatory structure requiring essential contributions from multiple regions of the AE2 TMD.

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The plasmalemmal SLC4 AE anion exchangers mediate electroneutral Na^+-independent Cl^-–HCO_3^- exchange to regulate intracellular pH (pH_i), intracellular $[Cl^-]$ and cell volume. Under usual physiological conditions, AE anion exchangers extrude HCO_3^- from cells, and load cells with acid and Cl^-. These processes are harnessed by polarized epithelial cells for transepithelial transport of salt, water and acid–base equivalents. Electroneutral Na^+-independent Cl^-–HCO_3^- exchange is mediated by at least three homologous, differentially expressed SLC4 gene products: SLC4A1/AE1, SLC4A2/AE2 and SLC4A3/AE3 (Alper, 2002; Romero *et al.* 2004). In contrast to the restricted distribution of AE1 predominantly in erythrocytes and in renal collecting duct Type A intercalated cells, the nonerythroid anion exchangers AE2 and AE3 are widely expressed in epithelial and other cell types. The SLC4 AE anion exchangers exhibit distinct patterns of acute regulation. In contrast to AE1, AE2 and AE3 are regulated by acute changes in pH_i (Stewart *et al.* 2001) and extracellular pH (pH_o) (Stewart *et al.* 2002). AE2 is also stimulated by NH_4^+ and hypertonicity via mechanisms requiring intracellular Ca^{2+} ($[Ca^{2+}]_i$), and by calmidazolium in a calmodulin-independent manner (Chernova *et al.* 2003). The structural basis for these regulatory differences between the closely related AE1 and

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AE2 anion exchanger polypeptides remains incompletely understood.

The SLC4 AE gene products AE1–3 share, along with other SLC4 transporters, a tripartite domain structure comprising a cytoplasmic N-terminal domain of 400–700 amino acids (aa), a transmembrane domain (TMD) of ∼500 aa that traverses the lipid bilayer 12–14 times, and a short C-terminal tail proposed to bind carbonic anhydrase II (Vince & Reithmeier, 2000). The orthologous TMDs of AE1–3 are ∼65% identical in aa sequence, with shorter regions of greater sequence conservation. In contrast, the orthologous N-terminal cytoplasmic domains share only ∼35% sequence identity. The AE TMDs expressed in erythrocyte membranes, in *Xenopus* oocytes or in HEK-293 cells suffice to mediate anion exchange (Grinstein *et al.* 1978; Kopito *et al.* 1989; Lindsey *et al.* 1990; Zhang *et al.* 1996). However, removal of the AE2 N-terminal domain alters regulation of AE2-mediated Cl^- transport by pH (Zhang *et al.* 1996; Stewart *et al.* 2001), NH_4^+, hypertonicity and calmidazolium (Chernova *et al.* 2003).

Whereas removal of most of the AE2 N-terminal cytoplasmic domain abolishes sensitivity to pH_i, the sensitivity of the remaining AE2 TMD to changes in pH_o is acid-shifted but not abolished. This indicates the presence within the AE2 TMD of amino acid residues that are required for normal regulation by pH. Our initial studies of the AE2 TMD have identified individual TMD His residues (Stewart *et al.* 2007*b*) and TMD charged residues which contribute to normal regulation of AE2 by pH (Stewart *et al.* 2007*a*). However, our inability to identify a single non-conserved amino acid residue responsible for pH sensitivity of AE2-mediated Cl^- transport suggests that multiple TMD residues comprise a 'pH sensor' in the AE2 transmembrane domain.

In the present study, we sought to identify subregions of the AE2 TMD required for normal regulation by pH_i and by pH_o, as well as to identify subregions important for regulation by NH_4^+ and by calmidazolium. To do so, we constructed a series of bipartite and tripartite chimeric AE2–AE1 transporters (see Fig. 1*A* (Stewart *et al.* 2007*a*) for sequence alignment). The encoded chimeric polypeptides were expressed in *Xenopus* oocytes and assayed for regulation of AE-mediated $^{36}Cl^-$ transport by pH_i, by pH_o, by NH_4^+ and by the calmodulin inhibitor, calmidazolium. We show that replacement of AE2 TMD residues in the large third extracellular loop (EC3) and at the TMD C-terminus with corresponding residues of AE1 alters minimally or not at all the AE2 regulatory phenotype for pH_i, pH_o and NH_4, whereas substitution of any other tested subregion of the AE2 TMD with corresponding AE1 sequence abrogates these responses. Thus, broad stretches of the AE2 TMD collaborate in this regulatory phenotype of AE2-mediated anion exchange. In contrast, the C-terminal 118 residues of AE2 are both necessary and sufficient to confer inhibition by calmidazolium upon the normally calmidazolium-insensitive AE1.

Methods

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

Construction of chimeric AE cDNAs

Plasmid pΔX (Alper *et al.* 1989) or plasmid pXT7 encoding mouse AE2 (Accession: J04036), and plasmid pBL encoding mouse erythroid AE1 (Accession no. X02677) or plasmid pL2AΔ encoding mouse kidney AE1 (Brosius *et al.* 1989) served as cDNA templates for polymerase chain reaction (PCR) and RNA transcription. Most chimeric cDNAs were constructed by the four primer PCR method previously described (Zhang *et al.* 1996; Chernova *et al.* 1997; Stewart *et al.* 2001), using the oligonucleotides (Biosynthesis, Woodlands, TX, USA), restriction enzymes and backbone acceptor fragment vectors listed in Supplemental Table 1. Integrity of PCR fragments and ligation sites was confirmed by DNA sequencing of both strands.

cRNA expression in *Xenopus* oocytes

Ovarian segments were excised from female *Xenopus laevis* (Xenopus One, Madison, WI, USA) anaesthetized with 0.17% tricaine according to protocols approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center. Minced ovarian fragments were incubated in 2 mg ml^{-1} Type A collagenase (Roche) for 1 h at room temperature in ND-96, pH 7.4, containing (mM): 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 Hepes, 2.5 sodium pyruvate and 5 mg (100 ml)$^{-1}$ gentamicin. Individual stage V–VI oocytes were manually defolliculated and injected on the same day with 50 nl cRNA or with H$_2$O. Capped mAE2, mAE1 and chimera cRNAs were transcribed from *Xba*I-linearized pXT7 or from *Cla*I-linearized pΔX templates with the T7 MEGAscript kit (Ambion, Austin, TX, USA), purified with the RNAeasy kit (Qiagen, Valencia, CA, USA), and resuspended in diethylpyrocarbonate-treated water. Formaldehyde agarose gel electrophoresis confirmed cRNA integrity, and cRNA concentration was estimated by A260 (Nanodrop, Wilmington, DE, USA). The amount of injected cRNA (0.5–25 ng) was titrated for each chimeric
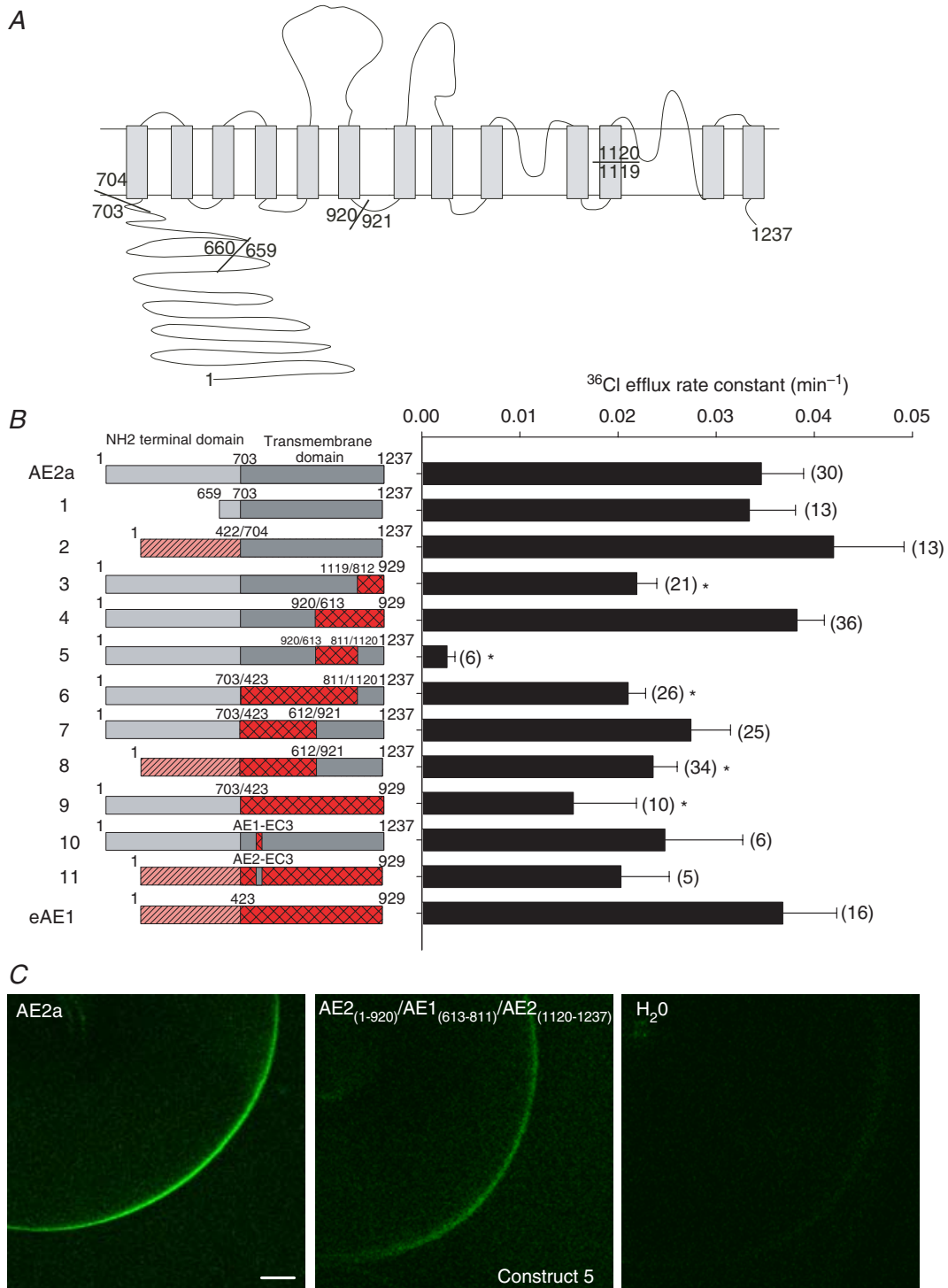
Figure 1. Chimeric and other mutant constructs studied

A, schematic of the AE2 TMD structure modelled on the human AE1 TMD topography proposed by Zhu *et al.* (2003), with mouse AE2a aa numbering (GenBank J04036) at N- and C-termini, and at junctions utilized for construction of chimeric and truncated cDNAs. *B*, wildtype, truncations and chimeric AE-mediated $^{36}Cl^-$ efflux from (*n*) *Xenopus* oocytes presented as rate constants (bar graph at right) for each numbered construct (schematics at left). Domains from mouse AE2a are light grey (N-terminal cytoplasmic domain) or dark grey (TMD). Domains from mouse eAE1 are hatched (N-terminal cytoplasmic domain) or cross-hatched (TMD). Amino acid numbers indicated at domain and/or chimera junctions. *$P < 0.05$ compared with wildtype AE2a. *C*, representative confocal fluorescence images of *Xenopus* oocytes expressing C-terminal GFP fusion proteins of wildtype AE2a (left), or the minimally active construct 5 (centre), or previously injected with water (right). Bar, 100 μm.

construct to approximate the $^{36}Cl^-$ efflux at pH_o 7.4 associated with injection of 10 ng wildtype AE2 cRNA. 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 experiments.

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

$^{36}Cl^-$ efflux assays were performed as previously described (Stewart *et al.* 2004, 2007*b*). Briefly, oocytes injected with 50 nl of 260 mm $Na^{36}Cl$ (15 000–20 000 c.p.m.) were allowed to recover for ∼10 min in Cl^-- free medium (mm: 96 sodium isethionate, 2 potassium gluconate, 1.8 calcium gluconate, 1 magnesium 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 (mm: 96 NaCl, 2KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, pH 7.4). 0.95 ml of this efflux solution were removed at 3 min intervals 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′-disulphonic acid (DIDS, 200 μm). Individual oocytes were lysed in 100 μl 1% sodium dodecyl sulphate (SDS) and lysates and efflux samples were subjected to scintillation counting for 3–4 min or until the magnitude of two s.d.s was < 5% of the sample mean.

$^{36}Cl^-$ efflux activity of tested chimeric AE polypeptides was compared with that of wildtype AE2 at pH_o 7.4 and to that of water-injected oocytes on each experimental day. Each cRNA construct was tested in oocytes harvested from at least two frogs. Experimental data were plotted as ln (% c.p.m. remaining in the oocyte) *versus* time. $^{36}Cl^-$ efflux rate constants were calculated from linear fits to data from at least three time points for each experimental condition. Injected cRNA quantities were titrated for wildtype AE2, AE1 and chimeric AE polypeptides to yield comparable ^{36}Cl efflux rate constants at pH_o 7.4. The amount of cRNA used for each construct is presented in Supplemental Table 3 together with complete $^{36}Cl^-$ efflux data for all experiments shown.

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 and validated (Stewart *et al.* 2004). AE2-mediated Cl^- transport sensitivity to pH_i at constant pH_o was tested by selective oocyte pH_i reduction (∼0.5 pH units, time constant = 403 ± 55 s, $n = 8$) with bath addition of 40 mm sodium butyrate (Stewart *et al.* 2001), followed by intracellular alkalinization (∼0.5 pH units, time constant = 315 ± 15 s, $n = 6$) accompanying removal of butyrate from the bath. $^{36}Cl^-$ 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. Butyrate is neither a substrate nor an inhibitor of AE2 (Stewart *et al.* 2001). AE2 sensitivity to pH_o at near-constant pH_i was measured by varying bath solution pH as previously described (Stewart *et al.* 2002).

Confocal laser immunofluorescence microscopy

The expression of AE chimeras at or near the oocyte surface was assessed in oocytes expressing AE chimera polypeptides fused at their carboxy-termini with Green Fluorescent Protein (GFP). At least eight oocytes expressing each AE–GFP fusion construct were fixed overnight in phosphate-buffered saline (PBS) containing 3% paraformaldehyde, washed three times in PBS, and stored at 4°C. Fixed, intact oocytes were imaged as whole-mount preparations with a Bio-Rad MRC1024 (Fig. 1*C*) or Zeiss LSM 510 laser scanning confocal microscope (Supplemental Fig. 1A) with a × 10 objective at constant pinhole, gain and filter settings. Representative images of whole-mount equatorial sections were compiled in Microsoft PowerPoint and oocytes exhibiting median levels of fluorescence intensity are shown in Fig. 1*C* and Supplemental Fig. 1A.

Surface polypeptide expression was correlated with $^{36}Cl^-$ efflux rate constant in oocytes previously injected with varying amounts of cRNA encoding wildtype AE2a–GFP. GFP fluorescence intensity at the oocyte surface (greyscale range 0–255 units) measured in four standardized regions of interest (width, 52 μm; height, 9 μm) in whole-mount confocal images (NIH ImageJ, http://rsb.info.nih.gov/nih-image/) was transferred into Microsoft Excel. After correction for background fluorescence intensity measured in oocytes previously injected with H_2O, mean surface pixel intensity was calculated for individual oocytes. Functional activities of wildtype AE2a–GFP were plotted against oocyte polypeptide surface expression across a wide range of injected cRNA quantities. The data were well described by a least squares linear fit ($r^2 = 0.98$), with saturation between 5 and 40 ng injected cRNA (Supplemental Fig. 1B).

Statistical analysis

Data are reported as mean ± s.e.m. Values for individual AE chimeras and groups of chimeras were compared with those of wildtype AE2 by Dunnett's 2-way *t* test, with significance defined as $P < 0.05$.

Results

Most chimeric AE1–AE2 polypeptides constructed are functional anion transporters

The C-terminal TMDs of murine AE1 and AE2 share ∼67% sequence identity (Alper, 1991) and mediate

comparable rates of $Cl^- - Cl^-$ exchange in the absence of most of their preceding cytoplasmic N-terminal domains (Zhang *et al.* 1996; Stewart *et al.* 2001). To define which portions of the AE2 TMD contribute to acute regulation of AE2-mediated anion transport, we constructed chimeras in which subdomains of the AE2 TMD were substituted with the corresponding TMD regions from the relatively pH-insensitive AE1 anion exchanger. Selection of junctions for the AE regions to be swapped (Fig. 1*A*) was guided by conserved unique restriction enzyme sites and by recent (Fujinaga *et al.* 1999) and current topographical models of human AE1 (Zhu *et al.* 2003). The conserved *BsrG*I site (AE2 aa 1119; AE1 aa 811) was used (Supplemental Table 1) to swap the C-terminal portions of the TMDs. The intracellular loop between putative TMDs 6 and 7 (AE2 aa 920/921) was chosen based on previous subdomain co-expression and deletion experiments for human AE1 (Groves & Tanner, 1999). With one exception, the AE chimeras exhibited $^{36}Cl^-$ efflux activity at pH_o 7.4 which was at least 50% those of wildtype AE2 and AE1. Only construct 5, $AE2_{(1-920)}-AE1_{(613-811)}-AE2_{(1120-1237)}$, was functionally impaired to the degree that regulation by pH_i and pH_o could not be accurately assessed (Fig. 1*B*). This loss of function correlated with reduced expression at or near the oocyte surface (26.5 ± 3.9 greyscale units ($n = 8$) for construct 5 *versus* 189 ± 12.2 greyscale units ($n = 6$) for wildtype or 14% of the wildtype level; Fig. 1*C*). Thus, reduced surface expression of construct 5 largely if not entirely accounted for its severely reduced transport activity.

Replacement of the AE2 EC3 sequence with that of AE1 does not alter AE2 regulation by pH_i or pH_o

We assessed the contribution to regulation of AE2 by pH of the poorly conserved sequence in the large exofacial loop connecting transmembrane spans 5 and 6 (EC3) by studying chimeras in which the EC3 sequence of mouse AE1 was inserted into mouse AE2 and vice versa (Fig. 2*A*). The resulting construct 10 ($AE2-AE1_{EC3}$) contains no N-glycans, whereas the complementary construct 11 ($AE1-AE2_{EC3}$) is predicted to contain the three N-glycans of the AE2 EC3 sequence plus the single N-glycan of AE1 EC4. Swapping EC3 loops between AE1 and AE2 had little effect on DIDS-sensitive $^{36}Cl^-$ efflux at pH_o 7.4 as compared with that of wildtype AE2 or AE1 (Basal $^{36}Cl^-$ efflux, Supplemental Table 3). As shown in Fig. 2*B* and summarized in Fig. 2*C*, chimera $AE2-AE1_{EC3}$ retained the wildtype AE2a phenotype of pH_i sensitivity, whereas chimera $AE1-AE2_{EC3}$ shared in this assay the pH_i insensitivity characteristic of wildtype AE1. Figure 2*D* compares the pH_o dependence of normalized $^{36}Cl^-$ efflux activity for these EC3 chimeras to those of wildtype AE2 and AE1. As summarized in Fig. 2*E*, the $pH_{o(50)}$ values

for wildtype AE2a and $AE2-AE1_{EC3}$ were comparable, and the indistinguishable $pH_{o(50)}$ values for wildtype AE1 and $AE1-AE2_{EC3}$ were acid-shifted to the same large extent ($P > 0.05$). Thus, the EC3 loop of AE2 is neither necessary for AE2 regulation by pH, nor sufficient to confer the AE2 pH-regulatory phenotype upon AE1. These data also demonstrate that N-glycosylation is not needed for functional expression of AE2 at the oocyte surface.

Replacement of any tested part of the AE2 TMD except C-terminal aa 1120–1237 with corresponding AE1 residues abolishes AE2 regulation by pH_i

Figure 3*A* shows $^{36}Cl^-$ efflux time courses from representative oocytes expressing wildtype or mutant AE polypeptides. The summarized efflux rate constants in the presence (black bars) and absence (grey bars) of 40 mM butyrate are presented in Fig. 3*B*. Figure 3*C* presents normalized pH_i sensitivity data for all tested chimeras. The cytoplasmic N-terminal domain of AE2 is required for wildtype regulation by pH_i (construct 1), consistent with our previous demonstration that conserved residues within that domain mediate this pH_i sensitivity (Stewart *et al.* 2002). The inhibition of wildtype AE2 by acidic pH_i was preserved (black bars) upon substitution of AE2 C-terminal aa 1120–1237 with the corresponding AE1 residues (construct 3) encoding a putative second re-entrant loop, two final trans-membrane spans and the short C-terminal cytoplasmic tail (Fig. 1*A*). AE2 pH_i sensitivity was preserved in attenuated form upon replacement of its N-terminal cytoplasmic domain with the shorter corresponding domain from AE1 (construct 2). All other tested chimeras (constructs 4–9) shared with wildtype AE1 a lack of inhibition by acidic pH_i (grey bars). These data show that non-conserved residues within the AE2 TMD separately encompassing aa 704–920 and aa 921–1119 are required for AE2 regulation by pH_i. However, substitution of AE2 aa 921–1237 into AE1 does not suffice to confer pH_i sensitivity on AE1. These data also confirm the ability of a substituted AE1 N-terminal cyto-plasmic domain to sustain regulation by pH_i when joined to the AE2 TMD (Stewart *et al.* 2001).

Replacement of any part of the AE2 TMD except aa 1120–1237 with corresponding AE1 residues severely acid-shifts or abolishes AE2 regulation by pH_o

Since pH_o regulates AE2 activity independently from pH_i (Stewart *et al.* 2002), we assessed the response of the AE2–AE1 chimeras to varying pH_o. Figure 4*A* shows that while replacement of AE2 N-terminal TMD residues 704–1119 with the corresponding AE1 residues 423–811 (construct 6) abolishes regulation of AE2-mediated $Cl^- - Cl^-$ exchange by pH_o, replacement of AE2 C-terminal

TMD residues 1120–1237 with the corresponding AE1 TMD residues 812–929 (construct 3) moderately alkali-shifts $pH_{o(50)}$ (7.24 ± 0.11, $n = 11$) from the wildtype AE2 value (6.89 ± 0.06, $n = 23$, $P < 0.05$). All other AE chimeras tested displayed either a greatly acid-shifted pH_o (constructs 3, 4 and 8) or, in the case of $AE2_{(1-703)}$–$AE1_{(423-811)}$–$AE2_{(1120-1237)}$ (construct 6), complete loss of inhibition by acidic pH_o (Fig. 4B). These data show that replacement of either the N- or C-terminal half of the AE2 TMD with corresponding residues from AE1 severely attenuates regulation by pH_o, and AE1 replacement of most of the AE2 TMD abolishes that regulation. However, near-normal or enhanced regulation by pH_o results from replacement of AE2 C-terminal aa 1120–1237 with corresponding AE1 residues and maintains near-normal anion exchange regulation by pH_o.
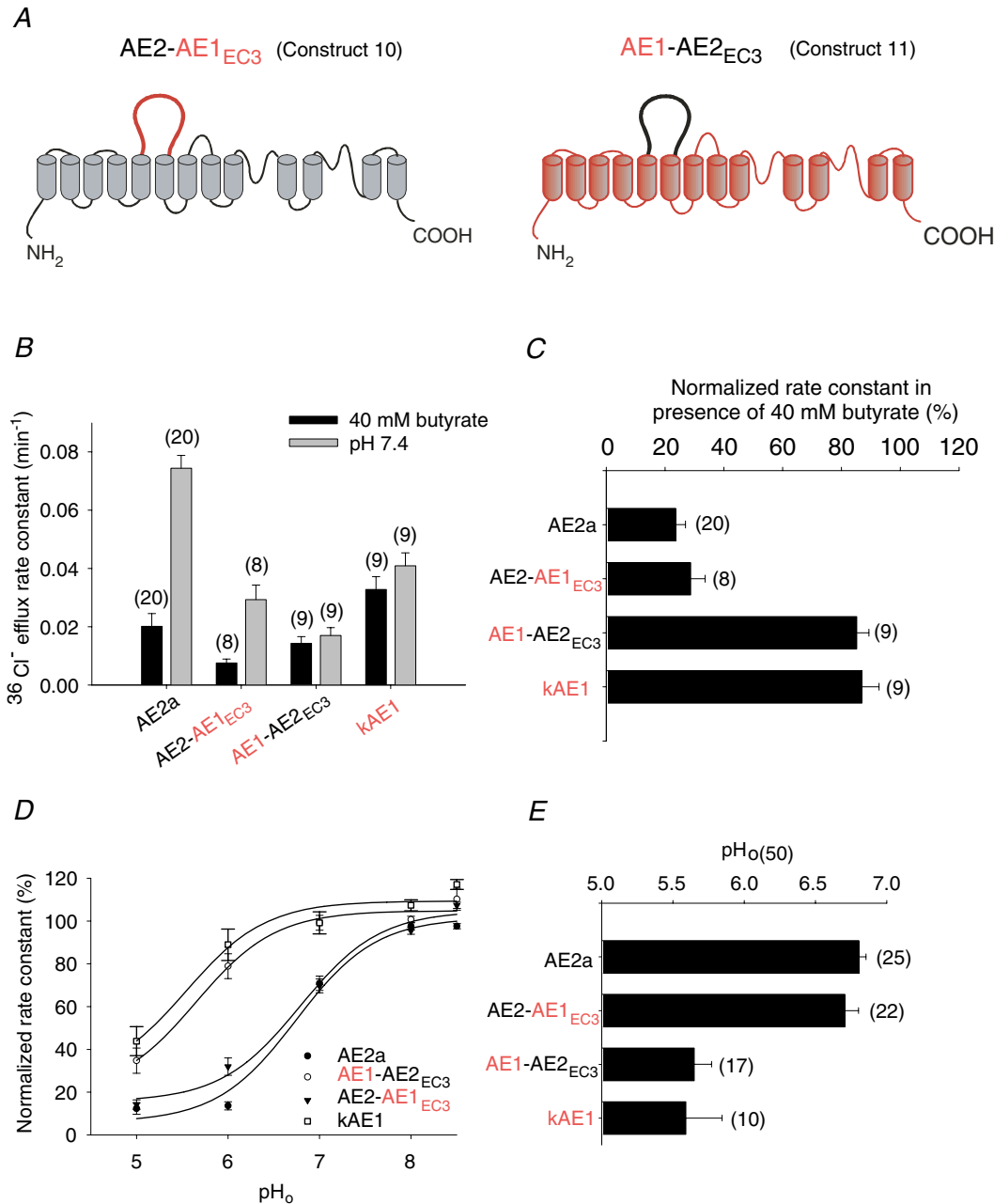

Figure 2. EC3 contribution to AE2 regulation by pH_i and pH_o
A, schematic of constructs 10 (AE2–AE1$_{EC3}$) and 11 (AE1–AE2$_{EC3}$). *B*, regulation of $^{36}Cl^-$ efflux by pH_i in (n) oocytes expressing the indicated AE polypeptides. *C*, normalized rate constants for the indicated AE polypeptides in (n) oocytes in the presence of butyrate (acidic pH_i). Interchange of EC3 residues does not alter regulation by pH_i. *D*, regulation of AE-mediated $^{36}Cl^-$ efflux at sequentially changing pH_o values. *E*, $pH_{o(50)}$ values in (n) oocytes expressing the indicated AE polypeptides. Interchange of EC3 residues does not alter regulation by pH_o.

Thus, those C-terminal AE2 residues within aa 1120–1237 that are not conserved in AE1 are not critical for regulation by either pH_o or pH_i.

Co-expression of separate, complementary AE2 sequences does not restore pH_i sensitivity to pH_i-insensitive AE1–AE2 chimeras or AE2 truncation mutants

We have shown previously that the $Cl^- - HCO_3^-$ exchange-incompetent mouse AE1 mutant E699Q can restore $Cl^- - HCO_3^-$ exchange activity to kAE1 mutants with missense or truncation mutations of the C-terminal cytoplasmic tail (Dahl *et al.* 2003) We therefore tested the hypothesis that the corresponding transport-incompetent AE2 mutants E1007Q and E1007K might restore pH_i sensitivity to pH_i-insensitive chimeric AE polypeptides (see Figs 3*C* and 5*A*) or to pH_i-insensitive AE2 cytoplasmic

N-terminal deletion mutants (Stewart *et al.* 2001). We also tested possible rescue of pH_i sensitivity by co-expressed pH_i-insensitive chimeric polypeptides of complementary sequence (Fig. 5*A*, construct 8 and construct 4). In view of the role of the AE2 N-terminal cytoplasmic domain in regulation by pH_i (Stewart *et al.* 2004), we further examined the hypothesis that co-expression of the N-terminal cytoplasmic and C-terminal transmembrane domains of AE2 as separate polypeptides might reconstitute pH_i-sensitive anion transport. Figure 5*A* summarizes the tested combinations.

Figure 5*B* shows that co-expression of the pH_i-insensitive chimera, $AE1_{(1-612)}-AE2_{(921-1237)}$ (construct 8) with the transport-inactive AE2 mutant E1007K did not restore regulation by pH_i. AE2 E1007K was similarly unable to rescue pH_i sensitivity of transport by the AE2 TMD ($AE2_{(660-1237)}$; construct 1) (Fig. 5*C*). Co-expression of the AE2 TMD ($AE2_{(660-1237)}$;
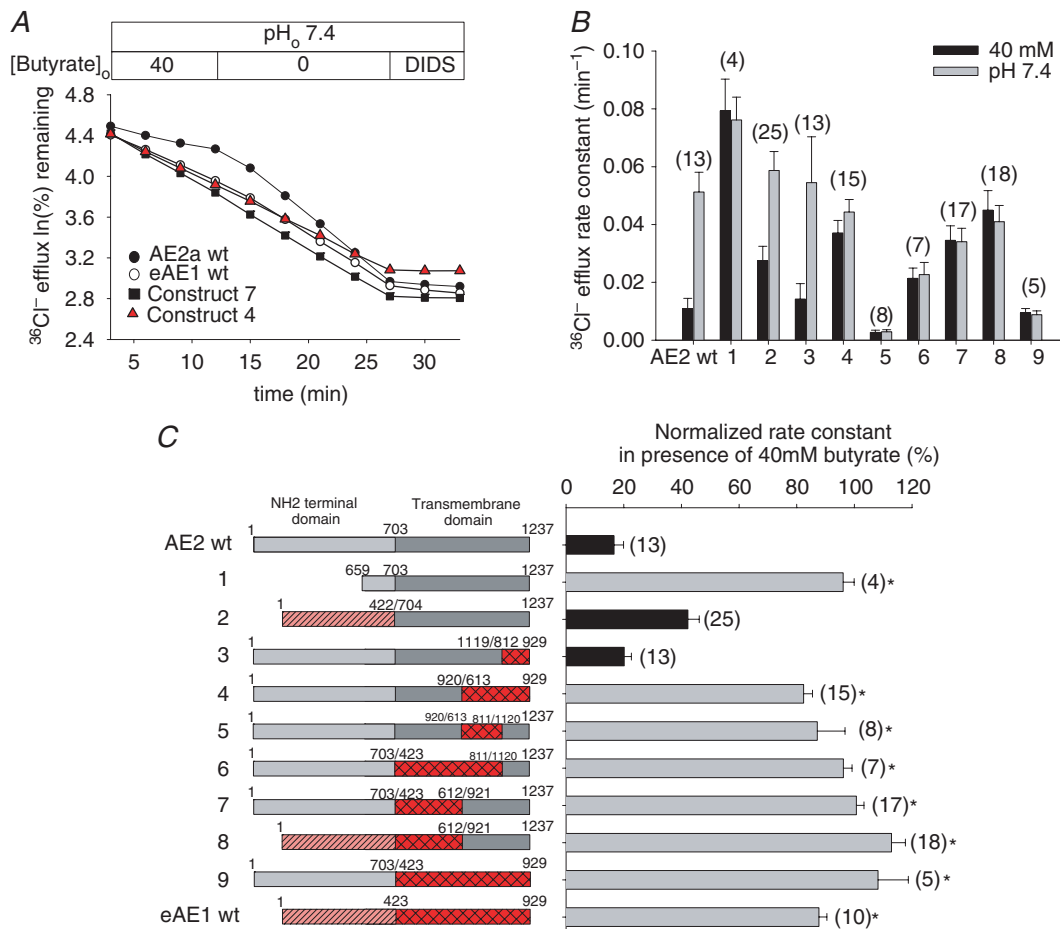

Figure 3. TMD contribution to AE2 regulation by pH_i
A, representative $^{36}Cl^-$ efflux traces from individual oocytes expressing the indicated AE polypeptides in the presence and subsequent absence of butyrate, followed by exposure to the inhibitor, DIDS. (See Methods for the effects of butyrate on pH_i.) *B*, $^{36}Cl^-$ efflux rate constants for (*n*) oocytes expressing the indicated wildtype or mutant AE polypeptides in the presence (black bars) and subsequent absence of butyrate (grey bars). *C*, normalized inhibition by butyrate of $^{36}Cl^-$ efflux (right) of (*n*) wildtype, mutant and chimeric AE polypeptides (schematics at left). Efflux was not significantly different (black bars) or was significantly different (grey bars) from wildtype AE2 (*$P < 0.05$).

construct 1) with its complementary N-terminal cyto-plasmic domain ($AE2_{(1-659)}$) also failed to restore pH_i sensitivity to AE2-mediated Cl^--Cl^- exchange (Fig. 5*B* and *C*). In addition, rescue of pH_i-sensitive $^{36}Cl^-$ efflux was unsuccessful with co-expression of complementary chimeras $AE1_{(1-612)}-AE2_{(921-1237)}$ and $AE2_{(1-920)}-AE1_{(613-929)}$ (constructs 8 and 4, Fig. 5*C*). The data provide no evidence under the conditions tested for the ability of residues on one AE monomer to confer pH_i sensitivity on a pH_i-insensitive second monomer within a heterodimer known to form in the context of AE1 (Dahl *et al.* 2003). Nor can the free AE2 N-terminal cytoplasmic domain rescue pH_i sensitivity of co-expressed AE2 TMD. AE2 regulation by pH_i may require intramolecular inter-actions.

Partial replacement of the AE2 TMD by corresponding AE1 residues attenuates or abolishes stimulation by NH_4^+

AE2 is acutely stimulated by NH_4^+ despite the fact that it acidifies the oocyte to pH_i values otherwise sufficient to inhibit activity completely (Humphreys *et al.* 1997; Stewart *et al.* 2001). This stimulation, an effect not shared by AE1, is inhibited by chelation of intracellular Ca^{2+} (Chernova *et al.* 2003; Kurschat *et al.* 2006). Our preliminary chimera studies showed a requirement for both the TMD and the N-terminal cytoplasmic domain of AE2 for NH_4^+-stimulated AE2 activity (Chernova *et al.* 2003). The AE TMD chimeras studied above have now allowed determination of those sections of AE2-specific TMD sequence required for NH_4^+ stimulation. Figure 6 shows that whereas wildtype AE2 and the chimera $AE2_{(1-1119)}-AE1_{(812-929)}$ (construct 3) were stimulated by 20 mм NH_4^+, the chimeric AE

constructs 4, 6, 7 and 8 resembled wildtype AE1 in their insensitivity to stimulation. Thus, although maximal NH_4^+ stimulation of AE2 appears to require the entire AE2 TMD, detectable stimulation is preserved when C-terminal AE2 aa 1120–1237 are substituted with the corresponding AE1 aa 812–929.

Partial replacement of the AE2 TMD by corresponding AE1 residues attenuates or abolishes inhibition by calmidazolium

The calmodulin inhibitor calmidazolium inhibits both basal and NH_4^+-stimulated AE2 activity, whereas AE1 is insensitive to the drug. However, other calmodulin antagonists and CaMKII inhibitors are not AE2 inhibitors. AE2 inhibition by calmidazolium does not require the presence of either the AE2 or the AE1 N-terminal cytoplasmic domains (Chernova *et al.* 2003). Figure 7 shows that replacement of the N-terminal half (construct 7) or three-quarters of the AE2 TMD (construct 6) with AE1 sequence, whether with or without AE1 substitution of the N-terminal cytoplasmic domain (construct 8), preserved the calmidazolium inhibition phenotype of wildtype AE2. This inhibition was attenuated but nonetheless sustained when AE1 sequence was substituted for the C-terminal half (construct 4) or quarter (construct 3) of the AE2 TMD. Although maximal inhibition of AE2 by calmidazolium may require other TMD residues, substantial inhibition is preserved when AE2 aa 1120–1237 (construct 3) or 921–1237 (construct 4) are substituted with the corresponding sections of the AE1 TMD. Thus, within the TMD, AE2 aa 1120–1237 are both necessary (construct 3) and sufficient (construct 6) for wildtype calmidazolium inhibition of AE2.
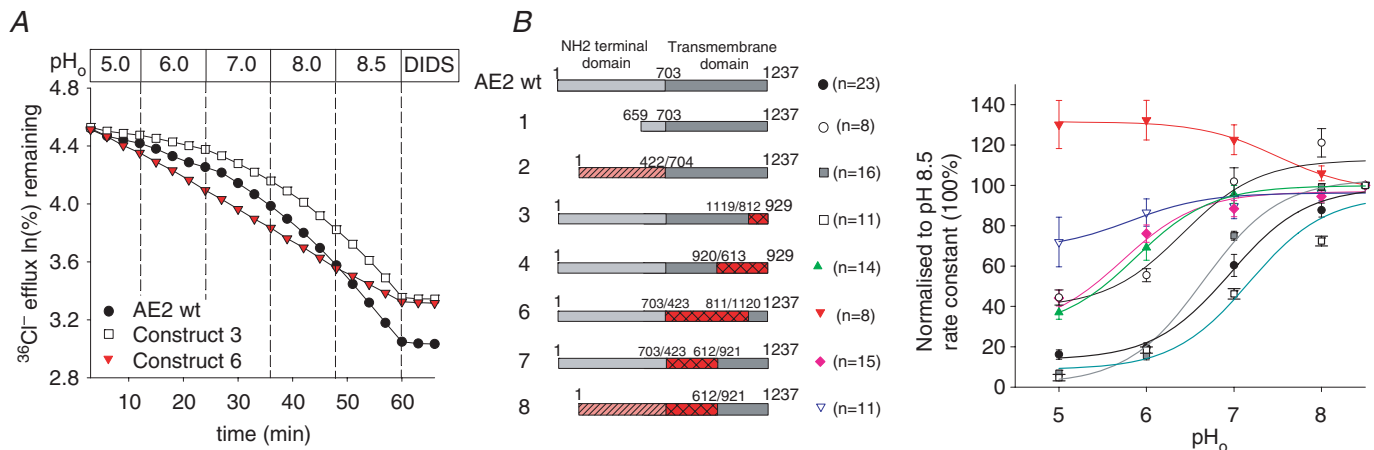

Figure 4. TMD contribution to AE2 regulation by pH_o
A, representative $^{36}Cl^-$ efflux traces from individual oocytes expressing the indicated AE polypeptides during sequential pH_o transitions from 5.0 to 8.5, followed by exposure to the inhibitor, DIDS. *B*, normalized pH_o dependence of $^{36}Cl^-$ efflux (right) by (*n*) oocytes expressing the indicated wildtype and chimeric AE2 polypeptides (numbered schematics and symbols at left).
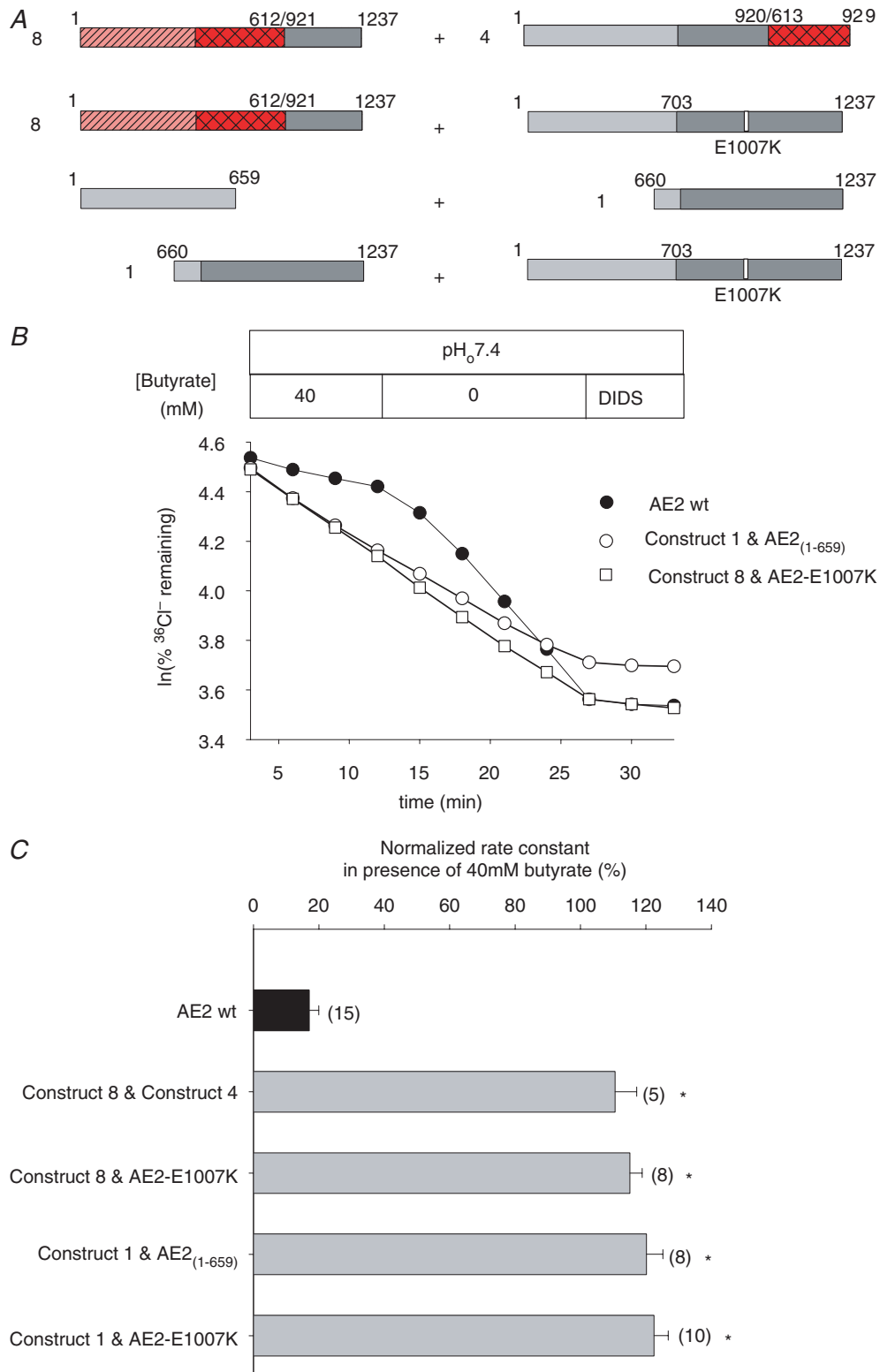
Figure 5. Loss of AE2 inhibition by acidic pH_i is not rescued by complementary sequences co-expressed in *trans*

A, schematics of co-expressed AE chimeras and mutants. *B*, representative $^{36}Cl^-$ efflux traces from individual oocytes expressing the indicated wildtype or mutant AE polypeptides in the presence and subsequent absence of butyrate, followed by DIDS inhibition. *C*, normalized rate constants in the presence of butyrate for the indicated wildtype and mutant AE polypeptides expressed in (*n*) oocytes. Grey bars indicate significant difference from wildtype AE2 (*$P < 0.05$).
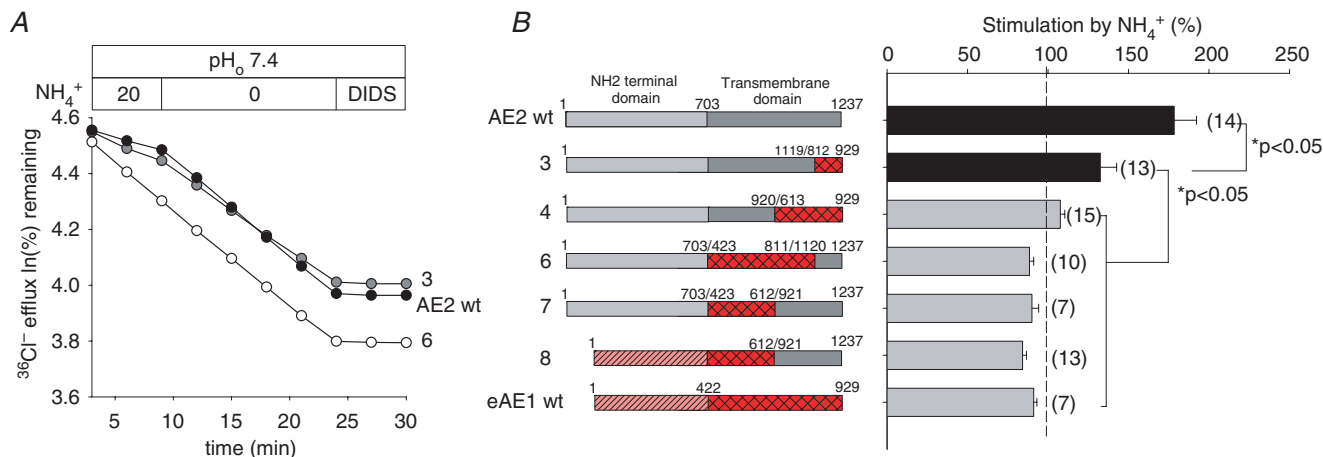
A

B

Figure 6. TMD contribution to AE2 stimulation by NH_4^+
A, representative $^{36}Cl^-$ efflux traces from individual oocytes expressing the indicated wildtype or mutant AE poly-peptides in the absence and subsequent presence of 20 mM NH_4^+, followed by DIDS inhibition. *B*, normalized stimulation by NH_4^+ in (*n*) oocytes (right) expressing the indicated wildtype and chimeric AE polypeptides (numbered schematics at left). Grey bars denote absence of stimulation.

Discussion

The structural basis for the distinct, acute regulatory properties of the SLC4 AE anion exchangers remains incompletely understood. We have shown with chimeric AE2–AE1 polypeptides that regulation of AE2 by pH_i and by pH_o requires AE2-specific amino acid residues from nearly the entire TMD sequence, with the exceptions of the third extracellular loop (Fig. 2) and of the C-terminal aa 1120–1237 (Figs 3 and 4) encompassing the last two putative transmembrane spans and the short C-terminal

cytoplasmic tail (construct 3). These data suggest that the TMD pH sensor is comprised of multiple amino acid residues within distinct TMD subdomains.

The entire AE2 TMD sequence was also required for full AE2 stimulation by NH_4^+ and for full inhibition by calmidazolium. However, substitution of AE2 aa 1120–1237 with the corresponding AE1 sequence (construct 3) preserved partial stimulation by NH_4^+ (Fig. 6) and partial inhibition by calmidazolium (Fig. 7). The presence of AE2 aa 1120–1237 as the only AE2 sequence in the TMD of the reciprocal chimeric construct
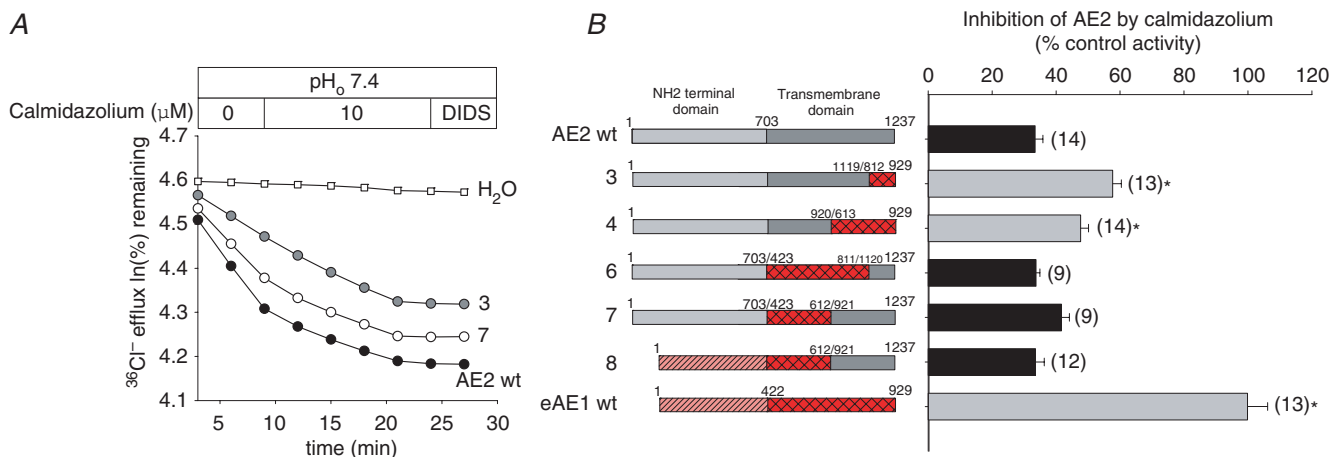
A

B

Figure 7. TMD contribution to AE2 inhibition by calmidazolium
A, representative $^{36}Cl^-$ efflux traces from individual oocytes expressing the indicated wildtype or mutant AE polypeptides in the absence and subsequent presence of 10 μM calmidazolium, followed by DIDS inhibition. *B*, normalized inhibition by calmidazolium of (*n*) oocytes (right) expressing the indicated wildtype and chimeric AE polypeptides (numbered schematics at left). Grey bars indicate significant differences from wildtype AE2 ($P < 0.05$).

6 sufficed to confer inhibition by calmidazolium on a chimera with its remaining TMD derived entirely from the calmidazolium-insensitive AE1.

Thus, the AE2-specific sequences within the third extracellular loop and within AE2 aa 1120–1237 are not required for transport inhibition by acidic pH, but are involved in wildtype regulation by NH_4^+ and are required for maximal inhibition by calmidazolium.

Anion transport function of AE2–AE1 chimeras

All but one of the chimeric constructs exhibited DIDS-sensitive Cl^-–Cl^- exchange activity within 2-fold of the transport activities of wildtype AE2 and AE1 (Fig. 1B). Although the low activity of construct 5 ($AE2_{(1-920)}$–$AE1_{(613-811)}$–$AE2_{(1120-1237)}$) precluded tests of inhibition by acidic pH_i, pH_o or calmidazolium, its activity remained DIDS sensitive. This low activity was explained in part by reduced surface expression (Fig. 1C). Thus (with the exception of construct 5), preservation of near-native polypeptide conformation tolerated considerable latitude in the placement of AE2–AE1 chimera junctions, suggesting only subtle differences in folding between the chimeric polypeptides and their wildtype parents. In contrast, TMD chimeras of AE2 with AE3 (which is processed to the HEK-293 cell surface 8-fold less efficiently than AE2) exhibit the AE3 TMD phenotype of low level surface expression (Fujinaga *et al.* 2003). Similarly, the mouse AE1 chimera in which substitution of putative TM spans 6–11 with the corresponding trout AE1 sequence led to loss of function probably due to absence from the oocyte surface (Borgese *et al.* 2004). Conformational studies utilizing introduced N-glycosylation sites engineered into human AE1 have suggested that both TM 12 and the N-terminal TMs 1–3 are both required for efficient membrane insertion of the first putative re-entrant loop of the AE1 TMD (Kanki *et al.* 2002). The interposed AE1 sequence in chimera 5 may thus alter interaction between these N-terminal and near C-terminal regions of the AE2 TMD, reducing anion transport to a greater degree than surface trafficking.

Structural subdomains of the AE2 TMD

The current study defines specific subdomains within the AE2 TMD that are required for normal regulation of anion transport. AE1 TMD subdomains are assembled during biosynthesis, and membrane insertion and trafficking as groups of transmembrane spans, based on the ability of co-expressed AE1 TMD subfragments to reconstitute $^{36}Cl^-$ influx (Groves & Tanner, 1999). The groups proposed were TM 1–5, TM 6–8, TM 9–12 and TM 13–14 (numbered according to a hydropathy-based topographical model of that time). TM 13–14 (hAE1 aa 825–911, roughly corresponding to AE2 aa 1120–1237

minus the putative re-entrant loop) was proposed to be placed on the periphery of the assembly of transmembrane spans, perhaps as a mobile element (Groves & Tanner, 1999). Choi *et al.* (2007) showed more recently that electrogenic Na^+–HCO_3^- cotransport requires interaction between two halves (TM 1–5 and TM 6-end) of the NBCe1 TMD. The chimera containing TM 1–5 from the electroneutral NBCn1 and TM 6-end from the electrogenic NBCe1 mediated electroneutral Na^+–HCO_3^- cotransport, implicating TM 1–5 of the TMD of NBCn1 as sufficient to confer electroneutrality. In contrast, both halves of the NBCe1 TMD were required for electrogenicity. The missense mutation experiments of Muller-Berger *et al.* (1995) led them to propose that TM 5, TM 8–10 and TM 13 contribute aa residues required for AE1-mediated Cl^- transport. All of these data are consistent with the observed requirement for most of the AE2 TMD to preserve wildtype AE2 patterns of acute regulation, and for the unique regulatory properties of AE2 aa 1120–1237.

Lack of evident contribution from inter-monomeric interactions to AE2 regulation by pH

The AE polypeptides form dimers or higher-order oligomers in the plasma membrane (Casey & Reithmeier, 1991; Zolotarev *et al.* 1999; Taylor *et al.* 2001). However, a single functional AE1 monomer within a dimer suffices to mediate Cl^-–HCO_3^- exchange in erythrocytes (Jennings & Gosselink, 1995) and *Xenopus* oocytes (Dahl *et al.* 2003). Functional evidence nevertheless suggests interaction between mutant and wildtype AE1 monomers within heteroligomers (Kuma *et al.* 2002; Cordat *et al.* 2006). We tested here the possibility that pH sensitivity can be conferred on a pH-insensitive but functional monomer by a co-expressed, Cl^- transport-incompetent AE2 variant. However, loss of pH regulatory function was not successfully complemented *in trans* by complementary AE2 sequence in the context of either co-expressed chimeras or AE2 subdomains (Fig. 5). Thus, these results provide no evidence for an intermonomeric component of AE2 regulation by pH.

Lack of regulatory function of AE2 N-glycans

N-glycosylation is important for normal trafficking or function for some membrane proteins. Thus, engineered or enzymatic lack of N-glycosylation in the GAT1 GABA–Na^+–Cl^- cotransporter leads to reduced protein stability, surface delivery, Na^+ affinity and slowed catalytic cycling (Cai *et al.* 2005). Mutation of N-glycosylation sites in the Fe^{2+}–H^+ cotransporter DMT1 prevents sorting to the apical membrane (Tabuchi *et al.* 2002). Moreover, disruption of N-glycosylation at N39 of the Cl^--dependent (Rizwan *et al.* 2007)

organic anion exchanger hOAT1 abolishes transport activity without altering surface expression, but mutation of all N-glycosylation sites also impairs surface delivery (Tanaka *et al.* 2004). However, as AE2–AE1$_{EC3}$ (construct 10) is devoid of N-glycans, whereas AE1–AE2$_{EC3}$ has four N-glycans (three from the EC3 of AE2 and one from the EC4 of AE1), the presence or absence of N-glycans on AE2 is not important for surface expression, function or regulation by pH in the *Xenopus* oocyte system (Fig. 2). These results parallel the lack of effect of tunicamycin treatment on either surface expression or function of AE2 expressed in HEK-293 cells (Fujinaga *et al.* 2003). Similar conclusions were reached for AE1 (Parker & Tanner, 2004) and for NBCe1 (Choi *et al.* 2003) expressed in *Xenopus* oocytes. The N-glycan of kAE1 could also be transferred from EC4 to EC3 without apparent consequence to trafficking in polarized MDCK cells (Quilty *et al.* 2002).

AE2 TMD regions important for regulation by pH

Replacement of C-terminal AE2 aa 1120–1237 with corresponding AE1 sequence (as in construct 3) is the only AE1 substitution besides that of EC3 which did not abrogate regulation of AE2 by pH$_i$. This distal region of AE2 includes the putative second re-entrant loop, the last two putative transmembrane spans that may include part of the anion selectivity filter (Zhu & Casey, 2004) and the C-terminal cytoplasmic tail encompassing a proposed binding site for carbonic anhydrase (Vince *et al.* 2000) (Figs 1 and 2). Replacement of this region by AE1 sequence slightly enhanced sensitivity to inhibition by extracellular protons (Fig. 4), whereas replacement of any other large section of AE2 TMD with the corresponding AE1 sequence abolished or greatly reduced sensitivity to inhibition by acidic pH on either side of the plasma membrane (Figs 3 and 4). Thus, AE2 TMD aa 703–1119 are required for regulation by both pH$_i$ and pH$_o$. We previously identified several individual aa residues of the AE2 TMD whose mutation alters AE2 regulation by pH (Stewart *et al.* 2007*a,b*). Some of these mutations selectively altered regulation by pH changes on only one side of the plasma membrane, but none individually constituted the complete 'pH sensor'. In addition, conversion of single amino acids within AE2 aa 1120–1237 to their AE1 counterparts led to enhanced proton sensitivity, consistent with the current data on the role of AE2 aa 1120–1237 in regulation by pH. These results further support the hypothesis that multiple amino acid residues located among several TM spans regulate AE2 activity in response to changing pH (Fig. 8 of Stewart *et al.* 2007*a*). They also provide further support for independent AE2 TMD sensors for pH$_i$ and for pH$_o$, but more precise definition of the proposed separate sensors will require additional

data. The minimal contribution of AE2 aa 1120–1237 to inhibition by acid pH does not arise from a greater degree of sequence identity with AE1 than is present in other tested TMD regions (Supplemental Table 2).

We have previously identified several conserved amino acids of the AE2 N-terminal cytoplasmic domain that, when mutated, dramatically alter regulation by pH$_o$ and by pH$_i$ (Stewart *et al.* 2004; Kurschat *et al.* 2006). We propose that such cytoplasmic domain residues interact with the TMD components of the pH sensor(s) to mediate inhibition of AE2 by protons. A similar interaction between a TMD cytoplasmic loop and C-terminal cytoplasmic tail residues has been proposed for the proton-mediated inhibition of connexin permeability (Duffy *et al.* 2002) additionally regulated by cytoplasmic CO_2 and aminosulphonates (Tao & Harris, 2004).

AE2 TMD regions important for stimulation by NH$_4$$^+$

Stimulation of AE2 by NH$_4$$^+$ also required integrity of the entire TMD (Fig. 6). However, replacement of AE2 aa 1120–1237 with the corresponding AE1 sequence was not entirely without effect, as observed for AE2 regulation by pH. Replacement of these residues attenuated but did not abolish AE2 stimulation by NH$_4$$^+$. Additional removal of TMD residues N-terminal to aa 1119 did abolish the stimulatory effect of NH$_4$$^+$. When AE2 aa 1120–1237 were substituted into a chimera with the remainder of its TMD from AE1 (Fig. 6, construct 6), these AE2 residues were not sufficient together with the AE2 N-terminal cytoplasmic domain to confer stimulation by NH$_4$$^+$. AE2 aa 1120–1237 may contribute to the action of the highly conserved aa 340–348 of the N-terminal cytoplasmic domain to mediate stimulation by NH$_4$$^+$ (Chernova *et al.* 2003). AE2 stimulation by NH$_4$$^+$ is attenuated by chelation of intracellular Ca^{2+}, but the mechanism by which NH$_4$$^+$ stimulates AE2 otherwise remains little understood.

AE2 TMD regions important for inhibition by calmidazolium

As observed for NH$_4$$^+$ stimulation, inhibition by calmidazolium was substantially attenuated but not abolished by substitution of AE2 aa 1120–1237 with corresponding AE1 sequence (Fig. 7, construct 3). However, attenuated inhibition by calmidazolium was preserved even when AE1 substitution extended to AE2 aa 921–1237, whereas stimulation by NH$_4$$^+$ was abolished. Another difference from the structural requirements of NH$_4$$^+$ stimulation was that AE2 aa 1120–1237 sufficed to confer calmidazolium inhibition onto construct 6, a chimera with all remaining TMD residues derived from AE1. Since the AE2 N-terminal cytoplasmic domain is not required for inhibition by

calmidazolium (Chernova *et al.* 2003), non-conserved residues present within AE2 aa 1120–1237 are probably the sole determinants of calmidazolium inhibition. As other inhibitors of calmodulin or of CaM-kinase II fail to replicate calmidazolium's inhibition of AE2 in *Xenopus* oocytes (Chernova *et al.* 2003), calmodulin-independent mechanisms of calmidazolium must be envisioned. These include direct inhibition of calcineurin and of adenylyl cyclases II and IX, activation of phospholipase A2, activation of Ca_i^{2+} entry through store-operated Ca^{2+} channels, inhibition of L-type Ca^{2+} channels and inhibition of acid secretion. These diverse actions might reflect calmidazolium's high affinity binding to the plasma membrane inner leaflet. The consequent reduction of inner leaflet negative surface charge may in turn decrease electrostatic membrane association of polycationic signalling proteins such as Kras, Src, MARCKS and the cytoplasmic tails of receptor tyrosine kinases. In addition, the calmidazolium-induced decrease in transmembrane electric field will be experienced as depolarization by intramembrane voltage sensors (Sengupta *et al.* 2007). Future experiments will determine whether calmidazolium inhibition can be localized to the C-terminal cytoplasmic tail or to the immediately preceding transmembrane spans of AE2.

Conclusion

Previous work has identified individual amino acids located across the entire AE2 TMD (both non-conserved and conserved in AE1) that are required for normal AE2 regulation by pH. However, no single amino acid residue substitution abolished regulation by pH, consistent with contributions of multiple TMD subdomains to the AE2 'pH sensor'. We have now identified subdomains of the AE2 TMD that are required for isoform-specific sensitivity to four types of acute regulation to which the related AE1 polypeptide is unresponsive. Normal inhibition of AE2 by acidic intracellular or extracellular pH and normal stimulation of transporter activity by NH_4^+ each require interaction between two TMD regions encompassing AE2 amino acids 703–843 and 920–1119. AE2-specific residues within C-terminal AE2 aa 1120–1237 are not critical for regulation by acidic pH and are not sufficient to confer normal stimulation by NH_4^+. In contrast, AE2 aa 1120–1237 are required for wildtype AE2 inhibition by calmidazolium, and substitution into the AE1 TMD of AE2 aa 1120–1237 suffices to confer full sensitivity to inhibition by calmidazolium. Future experiments will define more precisely the contributions of individual amino acid residues from each TMD subdomain to the complex TMD pH 'sensor(s)' that acutely regulate(s) AE2-mediated anion transport.

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Supplemental material

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