The Cytoplasmic and Transmembrane Domains of AE2 Both Contribute to Regulation of Anion Exchange by pH*

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We have compared regulation by pH of AE1 (band 3)- and AE2-mediated 36Cl− uptake into Xenopus oocytes. 36Cl− influx was assayed at varying extracellular pH (pHo) values between 9.0 and 5.0 under conditions in which corresponding intracellular pH (pHi) values were at or near steady-state. Wild type (WT) AE1 displayed a broad convex pH versus activity curve, with peak activity at pHi = 7.0 and 63% of maximal activity at pHi = 5.0. In contrast, WT AE2 displayed a steep pH versus activity curve, with peak activity at pHi = 9.0 and full suppression at pHi = 5.0. The structural basis of these differing pH sensitivities was examined by expression of cRNAs encoding chimeric and truncated proteins. Mutant polypeptides were expressed in oocytes and detected at the cell surface. The AE2cyto/AE1memb polypeptide displayed a broad pH versus activity curve similar to that of WT AE1. In contrast, the AE1cyto/AE2memb polypeptide displayed a steep pH versus activity curve, which was shifted toward acidic pH values from that of WT AE2 by 0.69 ± 0.04 pH units. Moreover, whereas the pH versus activity curves of AE2Δ99 and WT AE2 were indistinguishable, AE2Δ510 exhibited a pH versus activity curve acid-shifted from that of WT AE2 by 0.66 ± 0.13 pH units (indistinguishable from that of AE1cyto/AE2memb). The data suggest that a pH sensor resides within the transmembrane region of AE2. The affinity for protons of this pH sensor is influenced by a modifier site located between residues 99 and 510 of the N-terminal cytoplasmic domain of AE2. Acidification of oocytes with acetate suggested that pHi accounted for some but not all of the measured pH dependence of AE2.

The band 3-related AE anion exchanger gene family comprises at least three genes, each of which encodes polypeptides that mediate anion exchange when expressed in heterologous expression systems (reviewed in Ref. 1). The prototype red cell band 3 (AE1) serves both as a chloride/HCO3− exchange mechanism and as a major membrane anchor for the spectrin/ankyrin/actin cytoskeleton (2). The polypeptide products of the AE2 and AE3 genes are postulated to serve similar roles (1). Their polytopic transmembrane domains of ~530 C-terminal amino acids suffice to mediate anion exchange (3–5). Their N-terminal cytoplasmic domains of ~700 amino acids in length may, by analogy with red cell AE1, bind to cytoskeleton proteins of nonerythroid cells (6). Chloride/HCO3− exchange contributes widely to maintenance of cellular pH, to secondary active chloride loading, and to transepithelial movement of chloride and bicarbonate (1).

Whereas chloride/demonovalent anion exchange by red cell AE1 displays a broad pH versus activity profile (7), which serves to maintain relatively constant activity across the pH range of the capillary from its beginning to the end of its course through CO2− and acid-generating tissues, chloride/HCO3− exchange and 36Cl− influxes measured in tissue culture cells display a steep pH dependence (8–10). Tissue culture cells, as well as most nonerythroid cell types, generally express AE2 and/or AE3 ion exchangers rather than AE1 (1, 11–18). The steep pH dependence of the nonerythroid anion exchangers has led to the proposal that they contribute not only to cellular defense against alkaline loads, but also in some tissues to the maintenance of resting pHi (1, 19) as well as to maintenance of resting [Cl−] (20). However, when the pH dependence of AE2-mediated chloride/HCO3− exchange was tested in transiently transfected mammalian cells (21, 22), in infected insect cells (23), or in intact gastric glands (24), activation by alkaline pH, (when noted) required alkalization beyond 0.25 pH units. These findings suggested that AE2 might be inadequately sensitive to intracellular alkalization to play a physiological role in the regulation of resting pHi. In contrast, AE2 expressed in Xenopus oocytes was activated by exposure to alkaline media and inhibited by exposure to acidic media (25, 26). This pH dependence of AE2-mediated chloride/base exchange contrasted with AE1-mediated chloride transport which, in preliminary experiments, showed considerably less sensitivity to pH (46).

Heterologous expression of AE1 in mammalian cells has to date not allowed study of AE1 transport function at the cell surface (27). As with AE1, AE1/AE2 chimeric polypeptides also did not traffic to the cell surface. Study of microsomes from transfected cells (27) and of proteoliposomes prepared by reconstitution of these microsomes in the presence of exogenous phospholipid (28) has allowed comparison of sulfate fluxes mediated by heterologous AE1 and AE2. However, the sulfate/anion exchange measured in these studies is activated by acid pH, consistent with the proton/sulfate cotransport earlier described in red cells (29). The alkaline activation (or acid suppression) of AE-mediated chloride/base exchange in these vesicle systems has not been studied with the requisite time resolution. In contrast, AE1 is expressed at the surface of the Xenopus oocyte (30, 31) at least as efficiently as AE2 (25). Thus, the oocyte lends itself to the comparative study of the regulation of heterologous AE gene products in an intact cell system. We have compared the differences in pH dependence of chlo-

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ride/base exchange mediated by AE1 and by AE2 in Xenopus oocytes. The transmembrane domains of AE1 and AE2 are 65% identical in amino acid sequence. In contrast, the overlapping regions of N-terminal cytoplasmic domain are only 33% identical, and the 250 N-terminal amino acids of AE2 correspond to none in AE1. Inspection of the sequences suggests the possibility that the greater differences in the N-terminal cytoplasmic domains of AE1 and AE2 might explain the associated differences in pH sensitivity of chloride transport. However, analysis of the Na+/H+ exchanger, NHE1, provided evidence for a regulatory pH sensor in the transmembrane domain of the polypeptide. In addition, these authors localized a modulatory function to the cytoplasmic domain of NHE1. In order to define and localize structures in the AE2 polypeptide that mediate regulation of transport activity by pH, we expressed chimeric and truncated AE anion exchangers in Xenopus oocytes and assayed Cl– uptake and dNTPs were purchased from Promega (Madison, WI). Sure TM DNA polymerase. AE2 and AE1 underwent partial digestion with Sph I and complete digestion with Nae II and ligated to the large dIII fragment of pBL (AE1) and the 1.6-kb Stu I/HindIII fragment of pAE (AE2). The reconstructed AE2 cyto and AEmemb cDNA sequence encoded mouse AE2 cDNA from its translational initiation codon to nucleotide 1237 (corresponding to amino acids 1-422) fused to mouse AE2 cDNA from nucleotide 2292 to its 3’ end (corresponding to amino acids 704-1247, the entire transmembrane (TM)-spanning region beginning at TM1).

**EXPERIMENTAL PROCEDURES**

Materials—Female Xenopus were purchased from NASCO (Madison, WI) and maintained as described (25). 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Molecular Probes. Bumetanide was the gift of P. Feit (Leo Pharmaceutical). NaCl was purchased from ICN (Irvine, CA). All other chemical reagents of analytical grade and purchased from Sigma, Calbiochem, or Fluka. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Tag DNA polymerase and dNTPs were purchased from Promega (Madison, WI). Sure(TM) Escherichia coli strain was purchased from Stratagene (La Jolla, CA). Oligonucleotides were synthesized on a Milligen Cyclone Plus DNA synthesizer. Solutions—ND-96 medium consisted of (in mM) 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, 5 HEPES, and 2.5 sodium pyruvate. All flux media lacked pyruvate and contained 38 mM NaCl as NaAc. Some flux media substituted equimolar MES or bis-tris propane for HEPES. Flux media were titrated to the indicated pH values before use.

| Construct | Primer name | AE | Nucleotides (5’-3’) | Sequence |
|-----------|-------------|----|-------------------|----------|
| AE1cyto/AE2memb | yz5 | AE1 | 913-930 | 5’-GCCGTCCTCCTCGAGGAGC-3’ |
| | | AE2 | 2309-2292 | 5’-AATTAGCAAACACGGCAAGG-3’ |
| | | /AE1 | 1395-1378 | -CGTGGACTGAGCAGCCAT-3’ |
| | | /AE2 | 1378-1395 | 5’-CATGCGGCTCAGTCCCGCA-3’ |
| | | /yz2 | 2292-2309 | -TACGCGCTGCTGTTATA-3’ |
| | | /yz1 | 2651-2634 | 5’-AAAGATCTCTCTGGGTGAAL-3’ |
| | | /yz7 | 2058-2075 | 5’-CAACGACGATGCTAAAAG-3’ |
| | | /yz2 | 2143-1396 | 5’-GATGACAGACGACCAAGCAG-3’ |
| | | /yz3 | 2274-2291 | -CTGGGGGCTAAGTGCATT-3’ |
| | | /yz1 | 1396-1413 | 5’-GATGCCTAGGGCCACCCG-3’ |
| | | /yz7 | 2847-2829 | 5’-TCACCGCCCAACACTG-3’ |
| | | /yz1 | 480-496 | 5’-CCTCCCGCCGATCATG-3’ |
| | | /yz2 | 1106-1090 | 5’-GAGCAGGGAGGAAACC-3’ |
| | | /yz1 | 1714-1730 | 5’-TTCCCCGCGGATCATG-3’ |
| | | /yz2 | 2899-2881 | 5’-GAGCTACAGGTGCTT-3’ |

1 The abbreviations used are BCECF-AM, 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; WT, wild type; pH50, extracellular pH; pH1, intracellular pH; AE1cyto/AE2memb, cytoplasmic domain of AE2 attached to the transmembrane domain of AE1; AE1cyto/AE2memb, cytoplasmic domain of AE1 attached to the transmembrane domain of AE2; TM, transmembrane; bis-tris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane; HEPES, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid.
DNA Sequence Analysis—Sequencing reactions were carried out using the DyePrimer cycle sequencing kit (Applied Biosystems), and the reaction products were sequenced on the Applied Biosystems model 371 automated sequencer. PCR-amplified fragments and their ligation junctions in all reconstituted cDNAs were completely sequenced on both strands to verify absence of introduced mutations.

Inversion and Translation—cDNAs encoding WT AE1 and AE2cRNAAE1memb were linearized with HindIII. All other cDNAs were linearized with CiaI. Capped cDNA transcriptions were synthesized from these templates with the MEGAscript kit (Ambion, Austin, TX), and resuspended in diethylpyrocarbonate-treated water. RNA integrity was confirmed by formaldehyde gel electrophoresis, and concentration was determined by A260. In vitro translation of cDNAs in the absence and presence of dog pancreas microsomes was with rabbit reticulocyte lysate (Promega).

Antibodies—Affinity-purified polyclonal antibody to the 12 carboxy-terminal amino acids of murine AE2 has been described (16, 17). Murine monoclonal antibody to the 12 carboxy-terminal amino acids, 1224–1237, of murine AE2 was purified by fast protein liquid chromatography (Pharmacia Biotech Inc.) with protein A-agarose affinity chromatography. Polyclonal antiserum to the 12 carboxy-terminal amino acids of murine AE1 was the gift of Dr. A. M. Garcia.

Immunoprecipitation of Total and Surface AE Proteins from Xenopus Oocytes—Groups of 10–12 oocytes, injected either with 20 ng of cRNA or water, were incubated for 48 h at 19°C in ND-96, pH 7.4, containing 1 mCi/ml (30 μM) of 35S-methionine. Metabolically labeled oocytes were washed in modified ND-96, pH 8.0, in which 48 mM NaCl was replaced with 100 mM sucrose, and then incubated for 3 h at room temperature in the same medium in the presence or absence of 5 mg/ml papain. Subsequent washing, homogenization in Triton X-100, immunoprecipitation, and SDS-Polyacrylamide gel electrophoresis were as described previously (31).

[36Cl] Influx Measurements—Defolliculated oocytes were injected with cRNA or water, and incubated in ND-96 for 2–4 days at 19°C. Groups of 8–12 injected oocytes were preincubated for 15 min in 1 ml of ND-96 at the indicated pH and then transferred into 150 μl of the same medium containing in addition 10 μM bumetanide and 2.8–3.2 μCl of Na[36Cl]. Total NaCl concentration was constant at 96 μM (38 mM of which was from Na[36Cl]). Two 10-μl aliquots of mixed influx medium were removed for later determinations of [36Cl]-specific activity in each influx group. [36Cl] influx into oocytes was carried out for 15 or 60 min in the absence or presence, respectively, of 100 μM amiloride and then terminated by three washes in 25 ml of isotope-free ND-96. Each construct was tested under both influx conditions, with similar results.

Some oocytes were acetylated by acetate exposure. The incubation medium for these experiments was a modified ND-96 in which 38 mM NaCl was substituted with 38 mM sodium isethionate. Oocytes were preincubated for 30 min in this modified medium or in solutions in which sodium acetate was substituted for equimolar sodium isethionate. [36Cl] influx was then performed as above.

Individually injected oocytes underwent scintillation counting of associated [36Cl]. AE-mediated [36Cl] influx was calculated by subtracting values for Cl– influx into water-injected oocytes subjected to identical flux conditions as part of each experiment. Every experiment that tested chimeric constructs compared the chimeras with both WT AE1 and WT AE2 under the same experiment. Every experiment that tested AE2 truncation constructs compared the mutants with WT AE2 in the same experiment. Each construct was tested in oocytes from at least four frogs, using multiple RNA preparations.

AE-mediated [36Cl] influx values determined in flux media of pHo 5.0–9.0 were plotted as a function of pHo. Mean values measured for WT AE2, AE1cRNA/AE2memb, AE2Δ99, and AE2Δ510 in each individual experiment were fit to the following first-order logistic sigmoid equation using Ultrafit 2.1 (Eliezer): v = (Vmax × 10–k)/(10–k + 10–k), where v = measured AE-mediated Cl– influx, Vmax = the maximum value for AE-mediated Cl– influx, x = pHo of the experiment, and K = pH50, the pHo at which v is half-maximal. Results of individual experiments were pooled (see Figs. 5 and 6) by plotting data normalized to the fit parameters for each individual experiment. Differences in mean pH50 values of individual constructs (derived from curves of activity versus pHo) were subjected to statistical analysis by analysis of variance (34) and by two-tailed t tests (Microsoft Excel, version 5.0 for Macintosh). Results did not differ when influx assays were stratified according to performance of 15- or 60-min uptakes (n = 7 and n = 6, respectively, for WT AE2). Results also did not differ when influx assays were performed at 4 or 5 pHo values per experiment (n = 4 and n = 9, respectively for WT AE2). Therefore, pooled results were analyzed.

Mean values measured for WT AE1 and for AE2cRNA/AE1memb were also pooled by normalization to Vmax for each individual experiment. The transport values for these two polypeptides were not well fit by linear or logistic sigmoid functions and were plotted by hand.

Measurement of Oocyte Intracellular pH (pHi)—Oocyte pHi was measured by BCECF fluorescence ratio imaging as described previously (25, 26). Oocytes were loaded with 2–5 μM BCECF-AM for 1 h and mounted in a customized superfusion chamber mounted on an inverted microscope stage (25). Oocytes were irradiated, and fluorescence ratios were acquired and recorded to hard disk with an Image 1 digital ratio imaging system (Universal Imaging, West Chester, PA). The plane of focus of ~20 μm (~10 objective, N.A. 0.4) was set under the vegetal pole surface (25) for the measurements presented or at different focal planes (26) with similar results. In situ calibration of the BCECF fluorescence ratio (35, 36) was performed as described (25).

RESULTS

cDNA Constructs of AE Mutations—In order to localize those protein domains within AE2 and AE1 that are necessary for expression of their different sensitivities to pH, domain-swap chimeras and truncated AE2 cDNAs were generated as described under "Experimental Procedures" and in Table I. The constructs are presented in schematic form in Fig. 1. The AE1cRNA/AE2memb cDNA encoded a chimeric polypeptide consisting of mouse AE1 cytoplasmic amino acids 1–422 fused to mouse AE2 TM domain amino acids 704–1237. The AE2cRNA/AE1memb cDNA encoded a chimeric polypeptide consisting of mouse AE2 cytoplasmic amino acids 1–703 fused to mouse AE1 TM domain amino acids 423–929. AE2Δ99 cDNA encoded an engineered ATG followed by amino acids 100–1237. AE2Δ510 encoded an engineered ATG followed by amino acids 511–1237.

Biosynthesis and Surface Expression of WT and Mutant AE Polypeptides—cRNAs transcribed from the above described cDNAs were used as templates for in vitro translation reactions in reticulocyte lysate. Polypeptides of predicted size were translated from each cRNA. Each polypeptide was inserted into microsomal membranes and underwent an increase in M, and bandwidth suggestive of core N-glycosylation (not shown). Thus, the mutant cRNAs encoded polypeptides of the predicted length that folded in such a way as to permit core glycosylation.

Polypeptide biosynthesis was next analyzed in Xenopus oocytes (Fig. 2). Oocytes injected with WT AE2 cRNA produced polypeptides of 135 (core-glycosylated) and 160 kDa (complex-glycosylated) as shown previously (25), while WT AE1 cRNA produced the typical broad band at ~100 kDa, which contains within it both core- and complex-glycosylated species (30, 31). All constructs that contained the AE2 TM domain exhibited the
expected two polypeptide bands, whereas all those containing the AE1 TM domain displayed the expected single band. The truncated AE2 D99 cRNA produced polypeptides of 125 and 150 kDa, and D510 cRNA produced polypeptides of 80 and 115 kDa. Chimeric AE1cyto/AE2memb cRNA produced polypeptides of 110 and 135 kDa. Chimeric AE2cyto/AE1memb cRNA produced a single polypeptide of 130 kDa, which accumulated to the lowest level among the AE mutants tested.

The presence of these heterologous AE polypeptides at the oocyte surface was assessed by incubation of oocytes in the absence or presence of 5 mg/ml papain for 3 h at room temperature. Papain digestion of surface-exposed WT AE2 in Xenopus oocytes produced a carboxyl-terminal AE2 fragment of 32 kDa (Fig. 2). Accumulation of the 32-kDa fragment was maximal at 3 h. The same fragment was produced by papain digestion of porcine basolateral gastric microsomes, in which the amino acid sequence of the C-terminal fragment defined a 35-kDa fragment cleaved within the nonglycosylated ectoplasmic loop linking transmembrane spans 7 and 8. This papain treatment protocol did not increase 36Cl− influx in water-injected oocytes (not shown). Thus, papain digestion provided biochemical evidence for the presence at the oocyte surface of polypeptides encoding WT AE2, AE2D99, AE2D510, and AE1cyto/AE2memb.

The amount of AE2cyto/AE1memb at the oocyte surface was below the threshold of detection by polyclonal antibody to the carboxyl-terminal 12 amino acids of murine AE1. The corresponding carboxyl-terminal 35-kDa fragment of human AE1 produced by papain digestion of intact human red cell ghosts is barely detectable as a broad, very faint smear by Coomassie Blue stain (37). Although this fragment of WT human AE1 has been immunoprecipitated from metabolically labeled Xenopus oocytes with a particular monoclonal antibody (30), the comparable fragment from mouse AE1 has not yet been detected in Xenopus oocyte lysates. Nonetheless, expression of both WT mouse AE1 and AE2cyto/AE1memb lead to increased Cl− uptake, as shown below.

Functional Analysis of Expressed Mutant cDNAs in Xenopus Oocytes—Measurement of 36Cl− influx in oocytes expressing AE2cyto/AE1memb polypeptide provided a functional demonstration of surface expression of the mutant. Fig. 3 shows that injection of increasing mass quantities of AE2cyto/AE1memb cRNA produced increasing AE-mediated 36Cl− influx activity. However, the nmol of chloride uptake/ng of injected cRNA for this chimera, as also for AE2D510, was very low compared with those measured for WT AE2 and WT AE1. In contrast, AE2D99 and AE1cyto/AE2memb chimera were both considerably more active per ng of injected cRNA than either WT cRNA. Operationally defined specific activities of chloride transport among the cRNAs spanned a range of 150-fold, from 17.8 nmol/oocyte·h·ng (for AE1cyto/AE2memb) to 0.12 nmol/oocyte·h·ng (for AE2cyto/AE1memb and for AE2D510). These differences were replicated using cRNA from multiple transcription reactions and from multiple cDNA templates. The rank order of specific activities of the AE polypeptides tested correlated with the rank order of amounts of AE polypeptide detected at the surface by proteolytic cleavage (Fig. 2).

The considerable range of AE-mediated chloride uptake/ng of injected cRNA among WT and mutant AE polypeptides suggested that prior titration of functional activity would be im-
important for functional comparison of multiple cRNAs in different lots of oocytes. Dose-response curves such as those of Fig. 3 allowed selection of quantities of each cRNA for injection, which reliably yielded absolute transport rates differing by no more than 3-fold among the various cRNAs in individual experiments. In the experiments summarized in Figs. 5 and 6, maximal AE-mediated Cl⁻ uptakes at alkaline pH, were routinely 5–7 nmol/oocyte and never below 2 nmol/oocyte. The Cl⁻ uptakes measured for all AE polypeptide constructs in pH sensitivity experiments represented initial rates.

**Determination of Flux Assay Conditions in Which Oocyte pH Is Near Steady State—**When oocytes are placed into media of varied pH, pH changes in response. When pH₀ is shifted from neutral to acidic values, oocyte pH acidifies to a minimum and then recovers toward resting pH₁ (25). ~40% of this recovery is due to endogenous sodium/hydrogen exchange activity (26, 36, 38). We exploited these data to devise flux assay conditions in which AE-mediated chloride transport could be measured at near-steady-state oocyte pH values. pH₁ of individual oocytes expressing WT AE2 was monitored via BCECF fluorescence ratio imaging during the transition from ND-96, pH₀ 7.4, to preincubation medium of varying pH₀ for 15 min and then into flux medium of the same pH₀ containing in addition 100 μM amiloride and 10 μM bumetanide. As shown in Fig. 4, oocytes exposed to varying pH₀ under these conditions showed considerable pH₁ change within 15 min, achieved near-steady-state pH₁ values within ~30 min, and displayed minimal recovery over the ensuing 40–80 min (Fig. 4 and data not shown). At acid pH₀ this steady state reflected the balance between ongoing proton entry and the previously described (38) amiloride-resistant acid extrusion system(s) of the oocyte.3

Table II summarizes the near-steady-state pH₁ values attained in these conditions by oocytes exposed to extracellular media of the indicated pH₀ values. Steady-state pH₁ in the presence of amiloride changed 0.12 units/unit pH₀, similar to the value of 0.13 for maximal ΔpH previously determined in the absence of amiloride (25). This buffering of oocyte pH₁ contrasts with the minimal buffering of human erythrocyte pH₁ (Refs. 39 and 40; Table II), where the pH-dependence of AE1 measured previously reflected simultaneous and nearly equivalent variation of pH₁ and pH₀ (summarized in Ref. 7).

Regulation by pH of WT AE-mediated ³⁶Cl⁻ Uptake—**The pH dependence of chloride transport mediated by WT AE1 and by WT AE2 was compared under the assay conditions described in Fig. 4. Transport activities were measured at pH₀ values from 5.0 to 9.0. Near-steady-state pH₁ values measured in different oocytes in the same conditions ranged from 7.13 to 7.60 (Fig. 5). pH₀ was determined by glass pH electrode and by BCECF fluorometry did not change during oocyte incubations of 15 and 60 min (not shown). Nonetheless, since the pH₁ range chosen exceeded the useful pH range of HEPES buffer, identical experiments were also performed in media buffered by 5 mM MES and by 5 mM bis-tris propane. The results obtained did not differ from those measured in the presence of HEPES (not shown). In addition, all AE constructs were subjected to pH₀ versus activity assays in which 60-min ³⁶Cl⁻ influx in the presence of 100 μM amiloride was compared to 15-min ³⁶Cl⁻ influx in the absence of amiloride. pH dependence of transport in the two assay conditions did not differ.

WT AE1 and WT AE2 differed significantly in their regulation by pH (Fig. 5). WT AE1 (closed triangles) displayed a broad pH₀ versus transport activity curve (n = 6). Activity was maximal at pH₀ 7.0, decreased to 62.7 ± 6.9% of maximal value at pH₀ 5.0 (p < 0.005), and decreased to 82.7 ± 5.3% of maximal value at pH₀ 9.0 (p = 0.16). In contrast, WT AE2 activity (open triangles) displayed a steep dependence on pH₀. Activity was maximal at pH₀ 9.0 and decreased as pH₀ decreased, with a pH₀ at which activity was half-maximal (pH½) of 7.03 ± 0.08 (n = 13). AE2 activity was completely suppressed at pH₀ 5.0, approximately corresponding to pH₁ 7.13 (see Table II). The data relating pH₀ and AE2-mediated ³⁶Cl⁻ influx were well fit by a first order logistic sigmoid equation (Ultrafit goodness of fit index = 0.93 ± 0.03, n = 13).

Regulation by pH of Chimeric AE-mediated ³⁶Cl⁻ Uptake—**This major difference between pH₀ versus activity curves of WT AE2 and WT AE1 allowed us to test the hypothesis that a single region of AE2 distinct in amino acid sequence from AE1 is responsible for the difference. Therefore, the pair of domain-swap chimeras pictured in Fig. 1 was examined for pH sensitivity of ³⁶Cl⁻ uptake into oocytes. The hypothesis that the

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3 L. Jiang, M. N. Chernova, and S. L. Alper, unpublished results.
N-terminal cytoplasmic domain of AE2 was necessary and sufficient for the increased pH sensitivity of AE2-mediated $^{36}$Cl$^-$ uptake predicted in its simplest form that the AE2$_{cyto}$/AE1$_{memb}$ chimera would resemble AE2 in its regulation by pH. The converse hypothesis that the C-terminal transmembrane domain of AE2 was necessary and sufficient for the increased pH sensitivity of AE2-mediated $^{36}$Cl$^-$ uptake predicted in its simplest form that the AE1$_{cyto}$/AE2$_{memb}$ chimera would resemble AE2 in its regulation by pH. The experimental data partially supported this second hypothesis. The C-terminal transmembrane domain of AE2 proved necessary, but not sufficient, to reproduce the pH versus activity phenotype of WT AE2.

As shown in Fig. 5, AE2$_{cyto}$/AE1$_{memb}$ (closed circles) displayed peak activity at pH 8.0 and 9.0 and retained 61.9 ± 4.7% maximal activity at pH 5.0. (n = 4). This pH dependence of AE2$_{cyto}$/AE1$_{memb}$ resembled that of WT AE1 to a much greater degree than that of WT AE2. Therefore, the AE2 cytoplasmic domain was not sufficient to produce an AE2 pH-regulatory phenotype in this assay. In addition, the presence of the AE1 transmembrane domain sufficed to preserve most of the AE1 pH-regulatory phenotype.

The pH dependence of chloride transport activity of the converse chimera, AE1$_{cyto}$/AE2$_{memb}$, was also examined (open circles, Fig. 5). AE1$_{cyto}$/AE2$_{memb}$ almost precisely resembled WT AE2 in its pH$_i$ versus activity curve, except for a shift to acid pH values. AE1$_{cyto}$/AE2$_{memb}$ was also maximal at pH 8.0, but the pH$_{50}$ value was 6.34 ± 0.06 (n = 13). The mean of ΔpH$_{50}$ (the difference between pH$_{50}$ for WT AE2 and pH$_{50}$ for AE1$_{cyto}$/AE2$_{memb}$) values measured in each of 13 individual experiments was 0.69 ± 0.04 units (p < 0.001). This value and its statistical significance were not different, whether calculated only from the nine experiments in which transport was assayed at all five pH$_i$ values or (as presented) from these nine plus four additional experiments in which transport was assayed at only four pH$_i$ values. Results were also indistinguishable whether calculated as the mean of ΔpH$_{50}$ values calculated from individual experiments or as the ΔpH$_{50}$ calculated as the difference between mean pH$_{50}$ values for WT AE2 and for AE1$_{cyto}$/AE2$_{memb}$.

This result suggested that the AE2 carboxyl-terminal transmembrane domain contained a pH sensor sufficient for expression of most of the WT AE2 pH-regulatory phenotype. However, substitution of the AE1 cytoplasmic domain for that of AE2 in the presence of the AE2 transmembrane domain led to a decreased sensitivity to inhibition of transport activity by protons. Thus, the AE2 amino-terminal cytoplasmic domain exerted a modifier function that was necessary for complete replication of the WT AE2 pH-regulatory phenotype.

Regulation by pH of $^{36}$Cl$^-$ Uptake Mediated by N-terminally Truncated AE2 Polypeptides—The hypothesis of modifier function predicts in simplest form that sufficient truncation of the AE2 cytoplasmic domain should produce a pH versus activity phenotype that resembles that of the AE1$_{cyto}$/AE2$_{memb}$ chimera. In order to localize this proposed modifier function within the N-terminal cytoplasmic domain, two cytoplasmic domain truncation mutants of AE2 were examined. The pH dependence of AE2$_{N99}$ and AE2$_{N510}$ were assayed under the same experimental conditions as had been used to study the chimeric polypeptides. As shown in Fig. 6, the pH versus activity curves for these cytoplasmic domain truncation mutants were of similar shape to that of WT AE2, with complete inhibition of transport activity at pH 5.0. Whereas the pH dependence curve for AE2$_{N99}$ was indistinguishable from that of WT AE2, the curve for AE2$_{N510}$ was shifted to a more acidic pH value (ΔpH$_{50}$) between WT AE2 and AE2$_{N510}$ = 0.66 ± 0.13 (n = 5); p < 0.005). This result was unchanged when calculated as the difference between mean pH$_{50}$ values for WT AE2 and AE2$_{N510}$. The pH dependence curve of AE2$_{N510}$ did not differ detectably from that of AE1$_{cyto}$/AE2$_{memb}$. Thus the region between AE2 amino acids 100 and
Intracellular concentration of 13 mM neither acidified the oocytes nor inhibited AE2-mediated uptake of 36Cl\(^-\) (not shown). Thus, the inhibitory effect of extracellular acetate substitution was not likely due to simple inhibition of AE2 by competition at an anion substrate site but rather reflected an effect of intracellular acidification. Considered together, these data support the conclusion that pH\(_i\) contributed significantly, but not entirely, to the regulation of AE2 by pH under conditions in which both pH\(_i\) and pH\(_o\) were changed (as presented in Figs. 5 and 6).

### DISCUSSION

The present work has used analysis of recombinant chimeric and truncated polypeptides to initiate determination of the structural loci of pH sensitivity of AE2-mediated monovalent anion exchange. Functional studies of recombinant AE polypeptides have been reported in several heterologous expression systems. Although WT AE2 has been functionally expressed in mammalian cells (21, 25), heterologous WT AE1 and a version of the AE1\(_{cyt}/AE2_{memb}\) chimera did not reach the cell surface. In contrast, functional plasmaemal expression not only of AE2 (25, 26) but also of heterologous WT and truncated AE1 (30, 31, 41) has been reported in Xenopus oocytes. The present study establishes that Xenopus oocytes can also express functional chimeric AE polypeptides at the cell surface and so has set the stage for structure-function studies designed to delineate the amino acid sequences responsible for AE isoform-specific transport properties. We have begun this process with the study of the structural domains responsible for the different pH sensitivities of AE1 and AE2.

In contrast to the experiments that first demonstrated pH sensitivity of heterologous AE2 in Xenopus oocytes (25), the current flux assay was carried out at near-steady-state pH\(_i\). In the experiments of Figs. 5 and 6, oocytes were preincubated for 15 min at the desired pH\(_o\) prior to initiation of the 36Cl\(^-\) influx assay at the same pH\(_o\). Oocytes displayed minimal recovery from alkaline pH\(_i\) during the time course of the experiment in these nominally CO\(_2\)-free conditions. The presence of amiloride during 60-min influx assays to block the endogenous oocyte Na\(^+\)/H\(^+\) exchanger (26, 36, 38), or the performance of 15-min influx assays, achieved near-steady-state pH\(_i\) during influx periods. As pH\(_i\) was changed between 9.0 and 5.0, the corresponding steady-state oocyte pH\(_i\) values ranged from 7.60 to 7.13, respectively.

Injection of sodium acetate, pH 7.4, to an estimated final intracellular concentration of 13 mM neither acidified the oocytes nor inhibited AE2-mediated uptake of 36Cl\(^-\) (not shown). Thus, the inhibitory effect of extracellular acetate substitution was not likely due to simple inhibition of AE2 by competition at an anion substrate site but rather reflected an effect of intracellular acidification. Considered together, these data support the conclusion that pH\(_i\) contributed significantly, but not entirely, to the regulation of AE2 by pH under conditions in which both pH\(_i\) and pH\(_o\) were changed (as presented in Figs. 5 and 6).
The experiments demonstrated the requirement for the transmembrane domain of AE2 for display of the characteristic steep pH versus activity curve in WT AE2, AE1\textsubscript{cyto}/AE2\textsubscript{memb}, AE2\textsubscript{N},99, and AE2\textsubscript{N},510. In contrast, the presence in any polypeptide of the AE1 transmembrane domain produced a broad pH dependence of chloride uptake. Moreover, this pH sensitivity associated with the presence of the AE1 transmembrane domain was only minimally changed by substitution of the AE2 N-terminal cytoplasmic domain for its AE1 counterpart. This apparent lack of cytoplasmic domain specificity differed from the pH sensitivity of the AE2 transmembrane domain, which displayed modulation of affinity for proton equivalents by the contiguous N-terminal cytoplasmic domain of AE2, but not by that of AE1 (Fig. 5). Thus, attachment of the AE1 cytoplasmic domain onto the AE2 transmembrane domain led to an acid shift of 0.7 units in the pH\textsubscript{opt} value of the pH versus activity curve. This shift was reproduced by removal of the first 510 amino acids from the AE2 cytoplasmic domain. However, restricting the truncation to only 99 amino acids led to reproduction of the WT AE2 pH dependence phenotype (Fig. 6).

Definitive assignment of the AE2 transmembrane domain pH sensor to the endofacial or exofacial aspect of the plasmalemma is not yet possible. The combined variation of pH\textsubscript{i} and pH\textsubscript{o} did not allow unambiguous assignment of regulatory function to intra- or extracellular protons. Inhibition of AE2-mediated chloride uptake by acetate-induced intracellular acidification at constant pH\textsubscript{i} (Fig. 7) indeed suggested a role for pH\textsubscript{i} in this regulation. However, AE2 inhibition by acetate was accompanied by a reduction in pH\textsubscript{o}, of 0.16 units, whereas AE2 inhibition by extracellular acidification was accompanied by a 0.06-unit reduction of pH\textsubscript{i}. In addition, recent experiments have indicated that variation of pH\textsubscript{i} under conditions of minimal pH\textsubscript{o} change also can regulate AE2 activity.\textsuperscript{4}

Thus, pH\textsubscript{i} very likely could not account completely for the AE2 inhibition produced by lowering pH\textsubscript{o} in Figs. 5 and 6. These data together allow the proposal that AE2 can be regulated both by pH\textsubscript{i} and by pH\textsubscript{o}. Coincident variation of pH\textsubscript{i} and pH\textsubscript{o} is a common pathophysiologial derangement in the setting of ischemia and hypoxia and thus might cooperatively regulate AE2 activity in vivo.

When AE2 transport activity was plotted as a function of pH\textsubscript{o}, pH dependence fit simple logistic sigmoid kinetics. 80% of the range of measured AE2 activity was traversed across the 100-fold range of extracellular proton concentration between pH\textsubscript{o}, 6.0 and 8.0 (Fig. 5, upper x axis). pH\textsubscript{o}, could not be easily measured in the same oocytes in which influx measurements were performed, but pH\textsubscript{o} was measured in separate oocytes otherwise treated identically. When AE2 transport activity was plotted against the pH\textsubscript{i} values measured in identically treated oocytes (Fig. 5, lower x axis), 80% of the range of measured AE2 activity was traversed across a 2-fold range of intracellular proton concentration, between 28 and 51 nM (Table II and Fig. 5). This degree of cooperativity with respect to pH\textsubscript{i} allows one to account for a large part of the difference between the pH sensitivities of WT AE2 and WT AE1. In addition, the AE2 N-terminal cytoplasmic segment harbors a modifier domain whose removal reduces the proton affinity of the transmembrane domain pH sensor. The reported deletion experiments locate this modifier domain between mouse AE2 cytoplasmic amino acids 100 and 510 (Fig. 6). Whether the modifier directly recognizes the sensor or interacts with it indirectly or via additional polypeptide(s) remains to be determined.

The postulated pH-sensing function could have one or more of the following three topographical dispositions. The pH sensor might detect pH\textsubscript{i}, from the endofacial surface of AE2. Location of the sensor at the AE2 cytoplasmic face is suggested (but not required) by the cytoplasmic location of the modifier domain. Such a sensor might reside within cytoplasmic loops connecting transmembrane spans, within a water-accessible vestibule structure in the plane of the lipid bilayer, or in the C-terminal cytoplasmic tail. The same or a different pH sensor could detect pH\textsubscript{o} at exofacial residues, including a postulated vestibule structure such as that which might accommodate the sulfonate moieties of the stilbene inhibitors of transport (43). Alternatively, the sensor(s) might be located in a position that allows proton sensing of both intracellular and extracellular solutions, thus providing for dual sensing of pH\textsubscript{i} and pH\textsubscript{o}. Glu\textsuperscript{551} (44) and Lys\textsuperscript{551} (45) of human AE1 may be exposed to both sides of the red cell lipid bilayer. Such residues very likely contribute to the anion binding and translocation pathway through AE1.

In summary, AE2 and AE1 function in Xenopus oocytes differ in their regulation by pH. The steeper pH dependence of AE2 function is mediated by its C-terminal transmembrane domain. Modulation of that pH dependence requires a region of the AE2 N-terminal cytoplasmic domain (the modifier site) between residues 100 and 510. Future experiments will address the individual and cooperative roles of pH\textsubscript{i} and pH\textsubscript{o} in regulation of AE2 activity, define more precisely those residues that comprise the pH sensor(s) and modifier site of AE2, and examine the mechanisms by which these two functional domains interact with one another and with other regulatory sites within the AE2 polypeptide.

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