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Functional expression and subcellular localization of an anion exchanger cloned from choroid plexus

(intracellular pH/chloride transport/bicarbonate transport/epithelia/cerebrospinal fluid)

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ABSTRACT **We have isolated rat brain cDNA clones encoding AE2, a homologue of the erythrocyte anion exchanger, band 3 (AE1). Immunocytochemistry and *in situ* hybridization reveal that, in brain, AE2 expression is restricted to the basolateral membrane of the choroid plexus epithelium. Expression of a full-length mouse AE2 cDNA in COS-7 cells resulted in chloride- and bicarbonate-dependent alterations in intracellular pH, demonstrating that AE2 is a Cl/HCO_3 exchanger. Cation replacement studies indicate that AE2-mediated exchange is independent of extracellular sodium. COS-7 cells expressing a mutant rat AE2 cDNA clone that lacks the cytoplasmic NH_2-terminal 660 amino acids exhibit identical responses to cation and anion substitution. These results indicate that this domain does not play a significant role in either correct insertion of the transporter into the plasma membrane or anion exchange.**

Plasma membrane anion exchangers are a widely distributed class of transport proteins that play a key role in maintaining chloride and bicarbonate homeostasis within cells and in the extracellular fluid. The prototypical mammalian anion exchanger is the band 3 protein of the erythrocyte, encoded by the AE1 gene (reviewed in ref. 1). A distinctive feature of this glycoprotein is the presence of two distinct structural components: a soluble NH_2-terminal domain of $M_r \approx 45,000$ and a membrane-associated COOH-terminal glycosylated domain of $M_r \approx 65,000$ (reviewed in refs. 2, 3). The NH_2-terminal portion is exposed to the cytoplasmic face of the plasma membrane, where it associates with several cytosolic and cytoskeletal elements (reviewed in ref. 4). Most prominent among these interactions is the association with ankyrin, which serves to physically link the plasma membrane to the spectrin-based cytoskeleton (5). The ankyrin-binding site has not been mapped on the primary sequence of band 3.

The COOH-terminal half of band 3 comprises a domain that spans the phospholipid bilayer multiple times and participates in the transport of anions (2, 6, 7). Anion exchange mediated by band 3 is electroneutral, reversible, and inhibited competitively by disulfonic stilbene derivatives such as 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS), which can also covalently modify a single lysine residue within the COOH-terminal domain (8, 9). The prediction that this DIDS-binding residue corresponds to either Lys-539 or Lys-542 in the mouse band 3 sequence (10) has been recently confirmed by site-directed mutagenesis (11, 12). The putative membrane topology of this domain together with inhibitor binding and proteolytic dissection studies are all consistent with the interpretation that the COOH-terminal domain alone is sufficient to mediate anion exchange (2, 6, 7). Further, synchronized cell-free translation of band 3 mRNA indicated that insertion of the COOH-terminal half of the protein into the membrane proceeds in the absence of an NH_2-terminal signal sequence, implying that the COOH-terminal domain possesses the information necessary for correct insertion into the endoplasmic reticulum (13).

Band 3 (AE1) belongs to a family of homologous genes (reviewed in ref. 1) that includes AE2, a band 3-related cDNA previously cloned from erythroleukemic (14), renal, and lymphoid (15) cell lines, and a recently identified neuronal homologue, AE3 (16, 17). Comparison of the sequences of AE2 and AE3 indicates conservation of the overall domain organization noted above for AE1, with little primary sequence homology among the NH_2-terminal domains (1). By contrast, the COOH-terminal domains of AE2 and AE3, including the two potential DIDS-binding lysines, are highly homologous to the corresponding domain of AE1. This similarity has led to the speculation that AE2 is an anion exchanger that participates in the regulation of intracellular pH (pH_i) (15). Recently, such a function has been demonstrated for AE3, whose COOH-terminal domain shares about equal homology with AE2 and AE1 (16). Although transcripts of AE2 have been identified in a wide variety of epithelial and nonepithelial tissues (14, 15), there has been no direct assessment of the cellular or subcellular distribution of the AE2 polypeptide in any tissue. We have identified the sole site of AE2 expression in the brain and have used heterologous expression of AE2 cDNAs to directly assess its function in anion exchange.

MATERIALS AND METHODS

cDNA Cloning. AE2 cDNAs were isolated from a rat choroid plexus library (18) (kindly provided by D. Julius, University of California, San Francisco) screened at low stringency with a mouse AE1 probe (10). A full-length cDNA was assembled from two clones that overlapped at an internal *Eco*RI site and subcloned into Bluescript (Stratagene) vectors for amplification and sequencing. cDNAs were sequenced (19) on exonuclease III/S1 nuclease-generated nested deletions (20). cDNAs were also subcloned into the expression vector pMT2 (21) (generously provided by R. Kaufman, Genetics Institute).

***In Situ* Hybridization.** Bluescript plasmids bearing a 147-base-pair (bp) *Bgl* II/*Nsi* I fragment and a 173-bp *Alu* I/*Pst* I fragment isolated from the 3′ untranslated regions of mouse AE1 (10) and AE2 (15), respectively, were transcribed into antisense RNA probes using T7 polymerase (Stratagene) and UTP[^{35}S] (New England Nuclear). Brains from female BALB/c mice were perfused and fixed *in situ*, sectioned, and processed for *in situ* hybridization exactly as described (21).

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Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; pH_i, intracellular pH.

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Immunohistochemistry. Choroid plexus was freshly dissected from BALB/c mice and immediately fixed in paraformaldehyde/lysine/periodate as described (22). Cryostat sections (20 μm) were incubated with a 1:100 dilution of antiserum against an AE1 COOH-terminal dodecapeptide, α-C_t (22), which cross-reacts with AE2 (see below) (1:100). The immunoreactivity was detected with horseradish peroxidase-coupled sheep anti-rabbit IgG Fab fragments developed with diaminobenzidine in the presence of 0.01% H_2O_2 as described (22).

Studies on AE2 Transfected Cells. Choroid plexus was dissected from third and fourth ventricles of mouse brain and washed thoroughly in ice-cold PBS to remove erythrocytes prior to solubilization in SDS/PAGE sample buffer (23). COS-7 cells were transfected as described (16, 24). Cells were harvested by scraping after 48 hr and solubilized by incubation in 1% Triton X-100 at 4°C for 15 min. Detergent-insoluble material was removed by centrifugation for 10 min at 14,000 × *g* at 4°C (25). Protein samples were separated by SDS/PAGE, electrophoretically transferred to nitrocellulose, and blotted with α-C_t antibody followed by ^{125}I-labeled protein A as described (22). No bands were detected in parallel, identically processed blots incubated with nonimmune serum or immune serum in the presence of an excess of immunogen peptide (22).

pH_i measurements on transfected COS-7 cells, loaded with 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (26), were performed exactly as described (16).

RESULTS

The sequence of rat choroid plexus AE2 cDNA (Fig. 1*a*) is identical to that recently obtained for a rat stomach AE2 cDNA clone (17). The deduced amino acid sequence is 98.6%

a

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mouse:  MSSAPRRPASGADSLHTPEPESLSPGTPGFPEQ.EEDELRTLGVERFEEILQEAGSRGGEEPGRSYGEEDFEYHRQSSHHIHHPLSTHLPPDARRRKTPQGPGRKPRRRP 109
rat:    MSSAPRRPASGADSLHTPEPESLSPGTPGFPEQEEEDELRTLGVERFEEILQEAGSRGGEEPGRSYGEEDFEYHRQSSHHIHHPLSTHLPPDARRRKTPQGPGRKPRRRP 110
mouse:  GASPTGETPTIEEGEEDEEEASEAEGFRAPPQQPSPATTPSAVQFFLQEDEGAERKPERTSPSPPTQTPHQEAAPRASKGAQTGTLVEEMVAVASATAGGDDGGAAGRPL 219
rat:    GASPTGETPTIEEGEEDEDEVGEAEGFRAPPQQPSPASSPSAVQFFLQEDEGTDRKAERTSPSPPTQTPHQEAAPRASKGAQTGTLVEEMVAVASATAGGDDGGAAGRPL 220
mouse:  TKAQPGHRSYNLQERRRIGSMTGVEQALLPRVPTDESEAQTLATADLDLMKSHRFEDVPGVRRHLVRKNAKGSTQAAREGREPGPTPRARPRAPHKPHEVFVELNELLLD 329
rat:    TKAQPGHRSYNLQERRRIGSMTGVEQALLPRVPTDESEAQTLATADLDLMKSHRFEDVPGVRRHLVRKNAKGSTQAAREGREPGPTPRARPRAPHKPHEVFVELNELQLD 330
mouse:  KNQEPQWRETARWIKFEEDVEEETERWGKPHVASLSFRSLLELRRTLAHGAVLLDLDQQTLPGVAHQVVEQMVISDQIKAEDRANVLRALLLKHSHPSDEKEFSFPRNIS 439
rat:    KNQEPQWRETARWIKFEEDVEEETERWGKPHVASLSFRSLLELRRTLAHGAVLLDLDQQTLPGVAHQVVEQMVISDQIKAEDRANVLRALLLKHSHPSDEKEFSFPRNIS 440
mouse:  AGSLGSLLGHHHAQGTESDPHVTEPLIGGVPETRLEVDRERELPPPAPPAGITRSKSKHELKLLEKIPENAEATVVLVGCVEFLSRPTMAFVRLREAVELDAVLEVPVPV 549
rat:    AGSLGSLLGHHHAQGTESDPHVTEPLIGGVPETRLEVDRERELPPPAPPAGITRSKSKHELKLLEKI.ENAEATVVLVGCVEFLSRPTMAFVRLREAVELDAVLEVPVPV 550
mouse:  RFLFLLLGPSSANMDYHEIGRSISTLMSDKQFHEAAYLADERDDLLTAINAFLDCSVVLPPSEVQGEELLRSVAHFQRQMLKKREEQGRLLPPGAGLEPKSAQDKALLQM 659
rat:    RFLFLLLGPSSANMDYHEIGRSISTLMSDKQFHEAAYLADERDDLLTAINAFLDCSVVLPPSEVQGEELLRSVAHFQRQMLKKREEQGRLLPPGAGLEPKSAQDKALLQM 660
mouse:  VEVAGAAEDDPLRRTGRPFGGLIRDVRRRYPHYLSDFRDALDPQCLAAVIFIYFAALSPAITFGGLLGEKTKDLIGVSELIMSTALQGVVFCLLGAQPLLVIGFSGPLLV 769
rat:    VEVAGAAEDDPLRRTGRPFGGLIRDVRRRYPHYLSDFRDALDPQCLAAVIFIYFAALSPAITFGGLLGEKTQDLIGVSELIMSTALQGVIFCLLGAQPLLVIGFSGPLLV 770
mouse:  FEEAFFSFCSSNELEYLVGRVWIGFWLVFLALLMVALEGSFLVRFVSRFTQEIFAFLISLIFIYETFYKLIKIFQEHPLHGCSGSNDSEAGSSSSSNMTWATTILVPDNS 879
rat:    FEEAFFSFCKSNQLEYLVGRVWIGFWLVLLALLMVALEGSFLVRFVSRFTQEIFAFLISLIFIYETFYKLIKIFQEHPLHGCSVSNDSEAD.SSSNNMTWAATTLAPDNS 879
mouse:  SASGQSGQEKPRGQPNTALLSLVLMAGTFFIAFFLRKFKNSRFFPGRIRRVIGDFGVPIAILIMVLVDYSIEDTYTQKLSVPSGFSVTAPDKRGWVINPLGEKTPFPVWM 989
rat:    SA...SGQERPRGQPNTALLSLVLMAGTFFIAFFLRKFKNSRFFPGRIPGVIGDFGVPIAILIMVLVDYSIEDTYTQKLSVPSGFSVTAPDKRGWVINPLGEKTPFPVWM 986
mouse:  MVASLLPAVLVFILIFMETQITTLIISKKERMLQKGSGFHLDLLLIVAMGGICALFGLPWLAAATVRSVTHANALTVMSKAVAPGDKPKIQEVKEQRVTGLLVALLVGLS 1099
rat:    MVASLLPAVLVFILIFMETQITTLIISKKERIVQKGSGFHLDLLLIVAMGGICALFGLPWLAAATVRSVTHANALTVMSKAVAPGDKPKIQEVKEQRVTGLLVALLVGLS 1096
mouse:  MVIGDLLRQIPLAVLFGIFLYMGVTSLNGIQFYERLHLLLMPPKHHPDVTYVKKVRTMRMHLFTALQLLCLALLWAVMSTAASLAFPFILILTVPLRMVVLTRIFTEREM 1209
rat:    MVIGDLLRQIPLAVLFGIFLYMGVTSLNGIQFYERLHLLLMPPKHHPDVTYVKKVRTMRIDLFTALQLLCLALLWAVMSTAASLAFPFILILTVPLRMVVLTRIFTEREM 1206
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rat:    KCLDANEAEPVFDECEGVDEYNEMPMPV* 1235
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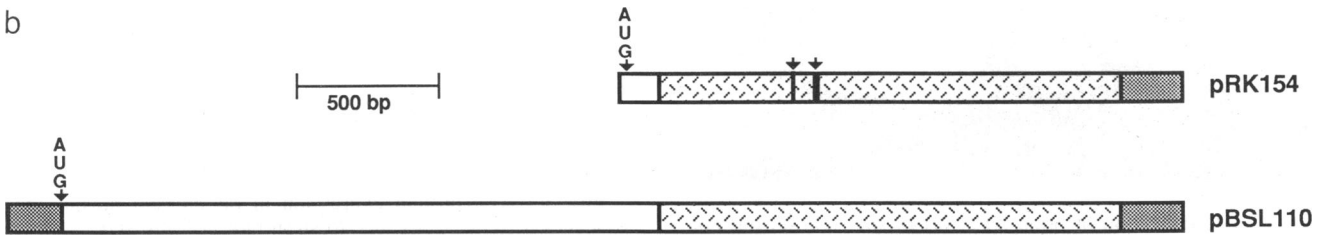

FIG. 1. AE2 cDNA clones. (*a*) Alignment of rat choroid plexus (upper line) and mouse lymphoid AE2 (15) amino acid sequences. The program GAP (27) was used for the alignment. (*b*) Map of the truncated rat AE2 cDNA (pRK154) and the mouse full-length AE2 (pBSL110) constructs used in the expression studies. Untranslated regions at the 3′ and 5′ ends are shown by the dotted shading. The NH_2-terminal region corresponding to the AE1 cytoplasmic domain is unshaded; hatched shading denotes the hydrophobic domain homologous to the AE1 membrane domain. Locations of two in-frame deletions of 3 and 9 bp, respectively, are shown by vertical arrows.

identical to murine AE2 clones isolated from kidney and lymphoid cells (Fig. 1*a*). The predicted rat AE2 protein is shorter than its murine counterpart by 3 amino acids. There are only 15 of a total of 1238 residues that differ between the two species. The rat sequence has a Glu at position 34 that is absent from mouse AE2. A tripeptide, Ser-Glu-Gln, and a Ser, present in the mouse sequence at positions at 882 and 861, respectively, are absent from the rat. Both of these differences occur in a region corresponding to the large extracellular loop of AE1 between putative transmembrane spans 5 and 6 (10). Indeed, 5 of the 15 amino acid differences between rat and mouse AE2 are located within the ≈55 residues comprising this putative extracellular loop, which is the least conserved region among all of the AE family members (1). Two constructs used in the transfection experiments are illustrated in Fig. 1*b*.

Antisense RNA probes corresponding to unique 3′ untranslated regions of mouse AE1 (10) and mouse AE2 (15) were used to examine sections of mouse brain by *in situ* hybridization at high stringency (21) (Fig. 2*a*). Hybridization with the AE1 probe in the brain was indistinguishable from that obtained with either probe in the "sense" orientation, consistent with Northern blot data (not shown), confirming the lack of AE1 gene expression in this tissue. In contrast, a strong signal was obtained with the AE2 probe exclusively in the choroid plexus of the third, fourth, and lateral ventricles (Fig. 2*a*, lower panel). Background levels of hybridization with this probe were observed in all other regions of the brain, indicating that choroid plexus is the sole site of AE2 expression. The localization of AE2 polypeptide exclusively in choroid plexus epithelium was confirmed by immunofluorescence staining of alternate serial sections (not shown) from the same mouse brain used in the above *in situ* analysis using a polyclonal antibody (α-C_t) to a highly conserved COOH-terminal synthetic peptide of mouse AE1 (22). Staining of thin sections of isolated choroid plexus with this antibody, which cross-reacts with AE2 (Fig. 2*b*), demonstrates that AE2 is localized exclusively at the basolateral plasma membrane of this polarized epithelium. No apical staining was observed. Examination of total choroid plexus protein by immunoblotting with α-C_t (Fig. 3*a*) revealed a single immunoreactive 165-kDa band (Fig. 3*a*, lane 2), demonstrating the absence from our preparation of erythrocyte AE1. This latter protein migrated as a characteristic broad smear of 95–105 kDa (Fig. 3*a*, lane 1). A 165-kDa band was also consistently observed in Western blots of choroid plexus from rat and cow (not shown), indicating that this epitope is highly conserved between species.

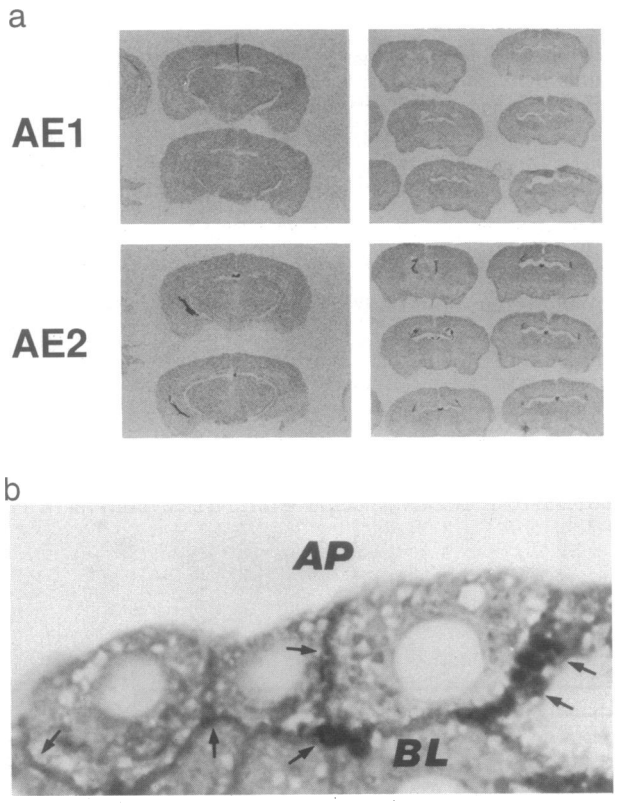

FIG. 2. Localization of brain AE2. (*a*) *In situ* hybridization of mouse brain using antisense RNA probes to mouse AE1 (upper panel) and AE2 (lower panel). (*b*) Immunoperoxidase localization of AE2 in thin section of mouse choroid plexus stained with α-C_t antibody. Apical (AP) and basolateral (BL) surfaces are noted. Arrows indicate basolateral membranes strongly stained with the antibody.

Two AE2 cDNA constructs (Fig. 1*b*) were expressed transiently in COS-7 cells. Plasmid pBSL110 contains the full-length murine lymphoid AE2 cDNA (15) (open reading frame = 1238 amino acids) and pRK154 contains a truncated rat choroid plexus AE2 cDNA. The open reading frame encoded by this latter plasmid initiates at Met-660, encoding a polypeptide that lacks all but the COOH-terminal 46 of the 705 NH_2-terminal amino acids of AE2, which are analogous to the cytoplasmic, membrane skeleton-binding domain of erythroid AE1 (28). Both constructs were efficiently expressed in COS-7 cells, as judged by Western blotting (Fig. 3). A major 165-kDa band was detected in detergent extracts of COS-7 cells transfected with full-length AE2 cDNA (Fig. 3*a*, lane 3) but not with vector alone (Fig. 3*a*, lane 4), demonstrating that the α-C_t antibody recognizes AE2. The mobility of AE2 from transfected COS-7 cells is indistinguishable from that observed in detergent extracts of native choroid plexus membranes (Fig. 3*a*, lane 2). A 155-kDa band, also detected in immunoblots of COS-7 cells expressing full-length AE2 (Fig. 3*a*, lane 3), probably represents an oligosaccharide processing intermediate, since it and the 165-kDa bands are replaced by a single 142-kDa species when AE2-transfected COS-7 cells are incubated for 24 hr in the presence of tunicamycin at 8 μg/ml (data not shown). Cells transfected with the truncated rat choroid plexus cDNA, pRK154, synthesized a major immunoreactive species of 65 kDa (Fig. 3*b*, lane 2). The molecular mass estimated for full-length and truncated AE2 polypeptides is in good agreement with the mass of the proteins predicted from the cDNA sequence. These mobilities are also consistent with the

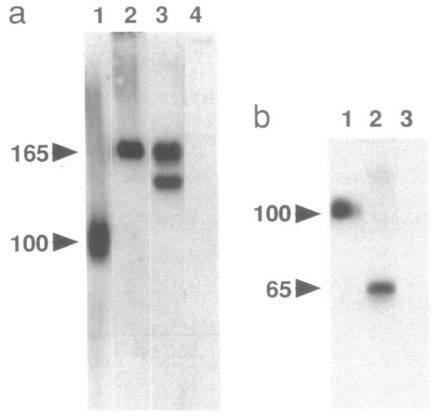

FIG. 3. AE2 protein expression in rat choroid plexus and in transfected COS-7 cells. (*a*) Western blot (7.5% PAGE) of human erythrocyte ghost membranes (lane 1); mouse choroid plexus (lane 2); COS-7 cells transfected with full-length AE2 cDNA, pBSL110 (lane 3) or vector, pMT2 (lane 4). (*b*) Western blot (10% PAGE) of erythrocyte ghosts (lane 1); COS-7 cells transfected with truncated AE2, pRK154 (lane 2) or vector, pMT2 (lane 3). Molecular masses are indicated in kDa.

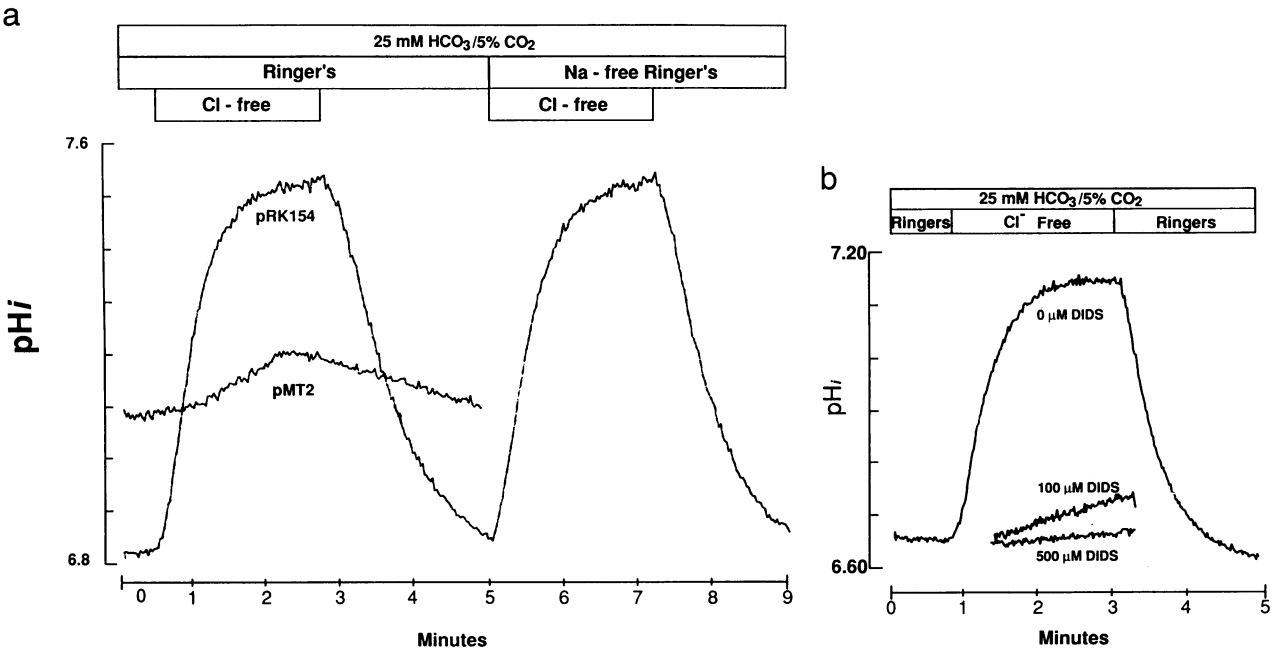

FIG. 4. pH_i measurements in single COS-7 cells expressing AE2 cDNA. (*a*) Effect of chloride and sodium removal on pH_i in cells transfected with AE2 cDNA (pRK154) or vector control (pMT2). (*b*) Effect of DIDS on Cl-dependent alkalinization in COS-7 cells transfected with pRK154. pH_i was recorded from a single cell treated as in *a* in the presence and absence of 100 μM DIDS. The trace with 500 μM DIDS was from a different, identically treated cell with the same resting pH_i and magnitude of Cl-free response. All traces are representative of at least 10 independent trials.

correct identification of the translational initiation sites in the sequences and with the presence in mouse (15) and rat AE2 of Asn-linked oligosaccharide chains.

To evaluate the function of AE2, we studied the effects of inhibitors and of ion substitution on pH_i regulation in transfected COS-7 cells. Typically, between 5% and 15% of cells transfected with either pRK154 or pBSL110 expressed AE2, as judged by immunofluorescence staining of fixed, permeabilized cells with α-C_t antiserum (data not shown). Individual transfected cells expressing AE2 were identified on the basis of their ability to mediate marked (>0.3 pH unit) intracellular alkalinization within 30 sec following Cl removal, a functional measure of anion exchange activity. COS-7 cells transfected with vector (pMT2) alone display a considerable variation in their response to this maneuver. The mean (±SEM) initial linear rate of pH_i increase following Cl substitution was 0.081 ± 0.03 pH/min, representing a range of 0.05–0.129 (n = 8). In contrast, cells selected as positive by our assay alkalinized at a mean rate of 0.857 ± 0.22 pH/min, with a range of 0.62–2.01. Importantly, no vector-transfected cells (n > 500) were observed to exhibit rates approaching the minimum criterion for selection as positive. Identification of functionally positive transfectants was also verified following pH_i study by immunofluorescence staining with α-C_t antiserum *in situ*. A total of 11 cells was examined in two different microscopic fields by the functional assay. The response of each cell and its position within the field were recorded. Without disturbing the microscope stage, the cells were fixed, permeabilized, and stained with antibody. The 4 cells that were scored as positive in the functional assay were all positive by immunofluorescence, whereas the 7 functionally negative cells were not immunoreactive.

The resting pH_i of cells expressing pRK154 (pH_i = 6.94 ± 0.03, n = 9) or pBSL110 (pH_i = 6.85 ± 0.03, n = 7) was significantly (P < 0.01, Student's two-tailed t test) more acidic than vector-transfected controls (pH_i = 7.10 ± 0.04, n = 7). Vector-transfected (Fig. 4*a*; pMT2) and wild-type (not shown) COS-7 cells respond to chloride removal with a variable but slow rise in pH_i, which is reversed upon readdition of Cl, consistent with the presence of a low level of background anion exchange activity. In contrast, COS-7 cells transfected with truncated (Fig. 4*a*; pRK154) or full-length (pBLS110; not shown) AE2 responded to removal of external Cl with a rapid and dramatic cytoplasmic alkalinization that was rapidly reversible upon return of Cl to the medium. Following return to baseline pH_i, Cl and Na were simultaneously removed. In six independent experiments, neither alkalinization due to Cl removal nor recovery upon restoration of Cl was dependent upon external Na. Alkalinization induced by Cl removal was partially inhibited in the presence of 100 μM DIDS (Fig. 4*b*), a blocker of anion exchange in erythroid (8) and nonerythroid (2) cells. Complete inhibition in this assay required the presence of DIDS concentrations in excess of 500 μM.

DISCUSSION

Our data demonstrate that AE2, which is expressed in a wide variety of cell types at the mRNA level (14, 15), functions as a Na-independent anion exchanger that is sensitive to inhibition by stilbenes. The concentration of DIDS required to completely inhibit AE2-mediated anion exchange in COS-7 cells is considerably higher than that required to block AE1 in erythrocytes (8). This relative insensitivity to DIDS has been previously observed for anion exchangers in a variety of nucleated cells, including human K562 (29), vero (30), HL-60 (31), and neutrophils (32, 33). In several of these and in other myelocytic lines (15, 29, 34), endogenous AE2 mRNA expression has been demonstrated, suggesting that this DIDS insensitivity might be an intrinsic property of AE2 and not a consequence of its cellular environment.

We describe a functional assay for identifying cells expressing AE2 cDNA, based upon the rapid alkalinization of the cell in response to removal of extracellular Cl. Such an alkalinization occurs as a consequence of reversal of the exchanger when provided with a gradient for Cl efflux and ample extracellular substrate (bicarbonate). It has been used by others (35, 36) as a criterion for identifying anion exchange in nonerythroid cells. Our data suggest that wild-type COS-7

cells express a low and variable level of anion exchange activity. However, the lack of immunological reactivity of vector-transfected COS-7 cells with the α-C_t antibody by Western blotting (Fig. 3) suggests that "basal" anion exchange activity is mediated either by a protein distinct from AE2 or by AE2 present at undetectably low levels. It has recently come to our attention that AE2 mRNA can be detected in COS cells by Northern blot analysis (S. L. Alper, personal communication), suggesting that this "background" anion exchange activity may well be due to low levels of expression of the endogenous gene. It is clear from Western blots (Fig. 3) and immunofluorescence (not shown), however, that AE2 expression in transfected cells exceeds the basal levels by several orders of magnitude (especially when considering that the Western blots compare the total AE2 levels in a population of transfected cells, of which only a fraction are actually positive). Similarly, we are confident that, although our functional assay is somewhat self-referential, overexpression of AE2 leads to Cl-dependent pH_i changes that exceed background levels in both rate and magnitude by severalfold.

The observation that resting pH_i in AE2-transfected COS-7 cells is lower than in vector-transfected controls is consistent with results we have previously reported for another band 3 homologue, AE3 (16). These data suggest that AE2 can function as an acid-loading anion exchanger, which, when overexpressed, surpasses the COS-7 cell's acid-extruding pH_i regulators. On the other hand, our data do not rule out the possibility that the transfection procedure itself could alter the COS-7 cell's ability to regulate pH_i, either by affecting the intrinsic buffering capacity or by altering the regulation of endogenous acid extruders. Although we can estimate the total number of AE2 molecules expressed per cell, our data do not allow us to estimate the number of these present at the cell surface. Differences in the copy number of functional (i.e., surface) AE2s could account for some of the observed variability in transport rates.

Previous studies of sulfate self-exchange in proteolyzed, resealed erythrocyte ghosts lacking the NH_2-terminal, cytoplasmic domain of AE1 suggested that anion transport activity is restricted to the COOH-terminal ≈45-kDa fragment (37). Those studies, however, assessed the role of AE1 proteolytic fragments in mediating divalent anion exchange, a process that differs in kinetic and mechanistic detail from the transport of physiologically relevant monovalent anions (2, 6). Moreover, the previous studies were unable to evaluate the role of the NH_2-terminal domain in mediating the correct insertion of the AE1 polypeptide into the plasma membrane. Our data confirm those observations with AE1 and extend them to AE2. We show that the 579 COOH-terminal amino acids of AE2 are sufficient for proper insertion of the protein into the plasma membrane and for mediating chloride/bicarbonate exchange activity. Further investigations are needed to determine whether the NH_2-terminal 659 amino acids play a role in regulating anion exchange activity, in targeting the nascent polypeptide to the correct membrane of epithelial cells or, by analogy with the corresponding domain of AE1, in tethering the plasma membrane to the epithelial cell spectrin/actin membrane skeleton.

Our data show that, in the brain, AE2 is expressed solely in the choroid plexus, an epithelium that is the major site of cerebrospinal fluid (CSF) production. The effect of stilbene disulfonates such as DIDS on the secretion of CSF (38, 39) and on its pH (40) has led to the proposal (41) that HCO_3 secretion into CSF is mediated by an apical plasma membrane anion exchanger. In contrast, Saito and Wright (38, 42) have proposed a model for HCO_3 secretion across the choroid plexus in which cyclic nucleotides activate an electrogenic efflux pathway for HCO_3 at the apical plasma membrane. They postulate that intracellular HCO_3 accumulation is achieved by the dissociation of intracellular carbonic acid and by DIDS-sensitive Cl/HCO_3 exchange at the basolateral membrane. Our data support the latter model and provide a molecular tool for future studies into the role of anion exchange in the formation of CSF.

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1. Kopito, R. R. (1990) *Int. Rev. Cytol.*, in press.
2. Knauf, P. A. (1986) in *Physiology of Membrane Disorders*, eds. Andreoli, T. E., Hoffman, J. F., Fanestil, D. D. & Schultz, S. G. (Plenum, New York), pp. 191–234.
3. Macara, I. G. & Cantley, L. C. (1983) in *Cell Membranes: Methods and Reviews*, eds. Elson, E., Frazier, W. & Glaser, L. (Plenum, New York), pp. 41–87.
4. Low, P. S. (1986) *Biochim. Biophys. Acta* **864**, 145–167.
5. Bennett, V. & Stenbuck, P. J. (1979) *Nature (London)* **280**, 468–473.
6. Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol.* **103**, 61–203.
7. Jay, D. & Cantley, L. (1986) *Annu. Rev. Biochem.* **55**, 511–538.
8. Cabantchik, Z. I., Knauf, P. A. & Rothstein, A. (1978) *Biochim. Biophys. Acta* **515**, 239–302.
9. Jennings, M. L. & Nicknish, J. S. (1984) *Biochemistry* **23**, 6432–6436.
10. Kopito, R. R. & Lodish, H. F. (1985) *Nature (London)* **316**, 234–238.
11. Bartel, D., Lepke, S., Layh-Schmitt, G., Legrum, B. & Passow, H. (1989) *EMBO J.* **8**, 3601–3609.
12. Garcia, A. M. & Lodish, H. F. (1989) *J. Biol. Chem.* **264**, 19607–19613.
13. Braell, W. A. & Lodish, H. F. (1982) *Cell* **28**, 23–31.
14. Demuth, D. R., Showe, L. C., Ballantine, M., Palumbo, A., Fraser, P. J., Cioe, L., Rovera, G. & Curtis, P. J. (1986) *EMBO J.* **5**, 1205–1214.
15. Alper, S. L., Kopito, R. R., Libresco, S. M. & Lodish, H. F. (1988) *J. Biol. Chem.* **263**, 17092–17099.
16. Kopito, R. R., Lee, B. S., Simmons, D. S., Lindsey, A. E., Morgans, C. W. & Schneider, K. (1989) *Cell* **59**, 927–937.
17. Kudrycki, K. E., Newman, P. R. & Shull, G. E. (1990) *J. Biol. Chem.* **265**, 462–471.
18. Julius, D., MacDermott, A. B., Axel, R. & Jessell, T. M. (1988) *Science* **241**, 558–564.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
20. Henikoff, S. (1984) *Gene* **28**, 351–359.
21. Simmons, D. M., Arriza, J. L. & Swanson, L. W. (1989) *J. Histotechnol.* **12**, 169–181.
22. Thomas, H. A., Machen, T. E., Smolka, A., Baron, R. & Kopito, R. R. (1989) *Am. J. Physiol.* **257**, C537–C544.
23. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
24. Oprian, D. D., Molday, R. S., Kaufman, R. J. & Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8874–8878.
25. Patel, V. P. & Lodish, H. F. (1987) *J. Cell Biol.* **105**, 3105–3118.
26. Rink, T. J., Tsien, R. Y. & Pozzan, T. (1982) *J. Cell Biol.* **95**, 189–196.
27. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
28. Bennett, V. (1985) *Annu. Rev. Biochem.* **54**, 273–304.
29. Law, F. Y., Steinfeld, R. & Knauf, P. A. (1983) *Am. J. Physiol.* **244**, C68–C74.
30. Olsnes, S., Ludt, J., Tonessen, T. I. & Sandvig, K. (1987) *J. Cell. Physiol.* **132**, 192–202.
31. Restrepo, D., Kozody, D. J., Spinelli, L. J. & Knauf, P. A. (1988) *J. Gen. Physiol.* **92**, 489–507.
32. Simchowitz, L. & Roos, A. (1985) *J. Gen. Physiol.* **85**, 443–470.
33. Simchowitz, L., Ratzlaff, R. & De Weer, P. (1986) *J. Gen. Physiol.* **88**, 195–217.
34. Restrepo, D., Kozody, D. J., Spinelli, L. J. & Knauf, P. A. (1989) *Am. J. Physiol.* **257**, C520–C527.
35. Vaughan-Jones, R. D. (1982) in *Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions*, eds. Nuccitelli, R. & Deamer, D. (Liss, New York), pp. 239–252.
36. Ganz, M. B., Boyarsky, G., Sterzel, R. B. & Boron, W. F. (1989) *Nature (London)* **337**, 648–651.
37. Grinstein, S., Ship, S. & Rothstein, A. (1978) *Biochim. Biophys. Acta* **507**, 294–304.
38. Saito, Y. & Wright, E. M. (1983) *J. Physiol. (London)* **336**, 635–648.
39. Frankel, H. & Kazemi, H. (1983) *J. Appl. Physiol.* **55**, 177–182.
40. Johanson, C. E., Parandoosh, Z. & Smith, Q. R. (1985) *Am. J. Physiol.* **249**, F478–F484.
41. Johanson, C. E. (1984) *J. Pharmacol. Exp. Ther.* **231**, 502–511.
42. Saito, Y. & Wright, E. M. (1984) *J. Physiol. (London)* **350**, 327–342.