The Extracellular Component of a Transport Metabolon

EXTRACELLULAR LOOP 4 OF THE HUMAN AE1 Cl⁻/HCO₃⁻ EXCHANGER BINDS CARBONIC ANHYDRASE IV*

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Cytosolic carbonic anhydrase II (CAII) and the cytoplasmic C-terminal tails of chloride/bicarbonate anion exchange (AE) proteins associate to form a bicarbonate transport metabolon, which maximizes the bicarbonate transport rate. To determine whether cell-surface-anchored carbonic anhydrase IV (CAIV) interacts with AE proteins to accelerate the bicarbonate transport rate, AE1-mediated bicarbonate transport was monitored in transfected HEK293 cells. Expression of the inactive CAII V143Y mutant blocked the interaction between endogenous cytosolic CAII and AE1, AE2, and AE3 and inhibited their transport activity (53 ± 3, 49 ± 10, and 35 ± 1% inhibition, respectively). However, in the presence of V143Y CAII, expression of CAIV restored full functional activity to AE1, AE2, and AE3 (AE1, 101 ± 3; AE2, 85 ± 5; AE3, 108 ± 1%). In Triton X-100 extracts of transfected HEK293 cells, resolved by sucrose gradient ultracentrifugation, CAIV recruitment to the position of AE1 suggested a physical interaction between CAIV and AE1. Gel overlay assays showed a specific interaction between CAIV and AE1, AE2, and AE3. Glutathione S-transferase pull-down assays revealed that the interaction between CAIV and AE1 occurs on the large fourth extracellular loop of AE1. We conclude that AE1 and CAIV interact on extracellular loop 4 of AE1, forming the extracellular component of a bicarbonate transport metabolon, which accelerates the rate of AE-mediated bicarbonate transport.

Carbonic anhydrases (CA)† (EC 4.2.1.1) are a family of zinc metalloenzymes that catalyze the rapid hydration/dehydration of CO₂/HCO₃⁻. Bicarbonate transport proteins are closely associated functionally with CA and together they eliminate the metabolic waste, CO₂, from the body. There are 14 mammalian isoforms of CA identified to date, varying in catalytic activity and tissue distribution (2–4). CAII, found predominantly in red blood cells, has been shown not only to bind to proteins of the AE family of Cl⁻/HCO₃⁻ anion exchange proteins but also to potentiate their transport activity by formation of a transport metabolon (5–8). A metabolon is a complex of proteins involved in a metabolic pathway that allows metabolites to move rapidly from one active site to the next (9, 10). The physical association of CAII with AE localizes the site of substrate (HCO₃⁻) production to the transport site, thus creating a transport metabolon. The CA-AE complex may also accelerate bicarbonate flux in part because of the increased CAII activity found upon interaction with its binding site on AE (11).

The AE family of proteins is comprised of AE1, AE2, and AE3 (12–15). The recently cloned AE4, although termed AE, shares little similarity with the other members of the AE family and is in fact more similar to the sodium/bicarbonate co-transporters (16). AE1 is expressed abundantly in erythrocytes and a truncated form is also present in the kidney and heart (17, 18). AE2 is almost ubiquitous, whereas AE3 expression is restricted to the brain, heart, and retina (13, 15, 19, 20).

Unlike cytosolic CAII, CAIV is anchored to the extracellular surface of the plasma membrane by a glycosylphosphatidylinositol anchor, thus reversibly hydrating CO₂ in the extracellular space (21). Northern blots, immunoblots, and immunohistochemical analysis, along with functional studies have localized CAIV expression to the heart, lung, kidney, brain, retina, and erythrocyte (22–31), all of which express AE proteins. CAIV hydrates CO₂ with a catalytic activity of 8 × 10⁵ s⁻¹, which is comparable with CAII (>10⁷ s⁻¹) (32). The two CA isoforms differ in their susceptibility to sulfonamide inhibitors, such as acetazolamide, with CAIV having an affinity up to 65-fold less than CAII (32). Furthermore, CAIV is unique in that it contains two disulfide bonds that contribute to its stability in 5% SDS, a concentration of denaturant that inactivates CAII (33).

The wide tissue distribution of AE proteins is mirrored by the broad expression of CA isoforms throughout the body. Whereas some tissues express only one CA isoform, other tissues express multiple isoforms. The extracellular CAIV isoform is expressed in the heart, but there is no evidence for cytosolic CAII (22, 34). Human erythrocytes express CAI, CAII, and CAIV (5, 31). The kidney, which avidly reabsorbs up to 500 g of NaHCO₃/day, expresses both membrane-bound CAIV and cytosolic CAII (24–27). CAII localizes to the cytosol of cells of renal tubules and collecting ducts where it is important for the acidification of urine (25), whereas membrane-bound CAIV localizes to the apical surface of cortical collecting ducts and α-intercalated cells (35). CAIV plays a major role in bicarbonate reabsorption by the kidney (36) as well as modulating the
pH in the tubule lumen (37). CAIV is also found on the surface of pulmonary endothelial cells (23) and in the endothelial cells of an ocular capillary bed, where its presence suggests it may be the target for CA inhibitors that are used in the treatment of glaucoma (30). Despite general knowledge of co-localization of carbonic anhydrases and bicarbonate transporters, precise structural inter-relationships have remained largely unknown. The physiological importance of bicarbonate metabolism and transport led us to investigate the physical and functional relationship between AE proteins and CAIV. In this study we found a functional interaction between AE proteins and CAIV. Expression of CAIV had no effect on the bicarbonate transport rate in cells expressing AE1 and cystolic CAII, because CAII maximizes the bicarbonate flux under these conditions. It was not possible to use inhibitors to block CAII function because any membrane-permeable CA inhibitor would access both extracellular CAIV and intracellular CAII. Thus we used a dominant-negative form of CAII to selectively neutralize the stimulatory effect of cytosolic CAII on AE transport activity and thereby examine the role of CAIV in AE-mediated bicarbonate transport activity. We found that like CAII, CAIV also accelerates AE-mediated bicarbonate transport activity. On the basis of co-migration on sucrose gradients, overlay assays, and GST pull-down assays we conclude that there is a physical association between extracellular CAIV and the integral membrane transport protein, AE1. The interaction occurs on the fourth extracellular loop of AE1. Taken together CAIV and AE functionally and physically interact to form the extracellular component of a bicarbonate transport metabolon, which potentiates AE-mediated bicarbonate transport.

EXPERIMENTAL PROCEDURES

Materials—ECL chemiluminescent reagent, donkey anti-rabbit IgG conjugated to horseradish peroxidase, and Hyperfilm were from Amer sham Biosciences. Poly-t-l-lysine and nigericin were from Sigma. Molecular Probes BCECF-AM was from Cascade Labs Limited (Canada). Glass coverslips were from Fisher. Jackson Immunoresearch Laboratories rabbit anti-goat conjugated to horseradish peroxidase was from BioCan Scientific (Mississauga, Canada).

Molecular Biology—An expression vector for the rabbit CAIV protein was received as a generous gift from George Schwartz (35), and Carol Fierke provided the V143Y CAII cDNA (38). Expression constructs for AE and CA proteins have been described previously (8–10). Plasmid DNA for transfections was prepared using Qiagen columns (Qiagen Inc., Mississauga, Canada).

Protein Expression—AE and CA proteins were expressed by transient transfection of HEK293 cells (42) using the calcium phosphate method (43). Cells were grown at 37 °C in an air/CO2 (19:1) environment in Dulbecco’s modified Eagle’s media supplemented with 5% (v/v) feto bovine serum and 5% (v/v) calf serum.

GST Fusion Protein Construction and Purification—Bacterial expression constructs encoding GST fusion proteins consisting of the cDNA for glutathione S-transferase fused to either cDNA corresponding to the third (amino acids 560–584, 5’-FGDPYLQSEYPVVMKPKPQGVPYP-3’) or fourth (amino acids 643–677, 5’-TTYKQLSGD-LKVSNSGHWIPGHLNYHFPK-3’) extracellular loop of rat AE1 were constructed. Using rat AE1 as a template, the forward and reverse primers, 5’-CCGGGATCTCAATGTTTCCAGCAGCCGTCTG-3’ and 5’-CCGGGATCTCAATGTTTCCAGCAGCCGTCTG-3’, respectively, were used in the same manner to produce the fourth extracellular loop product (GST-AE1C). The GST-AE1C3 and GST-AE1C4 constructs were verified by sequencing with a Beckman Instruments CEQ2000 DNA sequencer, and plasmid DNA was purified using Qiagen columns. The GST-AE1EC constructs were transformed into Escherichia coli BL21 and a single colony used to inoculate 50 ml of LB media. Following overnight growth at 37 °C with shaking this culture was used to inoculate 1.2 liters of LB media (5 ml/200 ml). The culture was grown at 37 °C with shaking until the A600 was 0.6–1.0. Isopropylthiogalactoside (1 mM final) was added and growth was allowed to continue for 2–6 h. The culture was then centrifuged at 10,000 × g for 10 min, and bacterial pellets were resuspended in cold PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4) containing protease inhibitors (complete Roche Molecular Biochemicals). Suspended cells were disrupted by sonication (4 × 1 min) and inoculated with Triton X-100 to a final concentration of 1% (v/v) with slow stirring for 30 min. Following centrifugation (15,000 × g, 10 min) the supernatant was transferred to glutathione-Sepharose 4B (50% slurry equilibrated with PBS) (Amersham Biosciences) and allowed to incubate on ice for 2 h. The sample was centrifuged (500 × g for 5 min) and the pellet was washed three times with PBS. The fusion proteins were eluted with glutathione buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0).

Immunodetection—HEK293 cells, grown in 60-mm tissue culture dishes, were transiently transfected with a construct, pJRCC (39), to induce expression of AE1 anion exchange protein as described above. Cells were also co-transfected with either pJRCS6 or pDS14 (8) to induce expression of human wild-type and mutant CAII, respectively, and also with a construct to express rabbit CAIV (35). Plasmids pBSL103 (40) and pJRCC1 (41) encoded mouse AE2 and rat AE3 cardiac, respectively. Two days post-transfection, cells were washed with PBS and lysates of the whole tissue culture cells were prepared by sonication of equal sample of SDS-M containing 150 mM Tris, pH 6.8) containing 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM 1-otosylamido-2-phenylamidochloromethyl ketone, 0.1 mM N′-tosyl-l-lysine chloromethyl ketone, and 2 mM EDTA. Prior to analysis, samples were heated to 65 °C for 5 min and sheared through a 26-gauge needle (BD PharMingen). Insoluble material was then sedimented by centrifugation at 16,000 × g for 10 min. Samples were resolved by SDS-PAGE on 8 or 12.5% acrylamide gels (44). Proteins were transferred to PVDF membranes by electrophoresis for 1 h at 100 V at room temperature in buffer composed of 20% (v/v) methanol, 25 mM Tris, and 1 mM EDTA. Prior to washing with TBST buffer, blots were incubated overnight in 10 ml of TBST-M containing 3 mM of 1858 rabbit anti-AE1 polyclonal antibody (46), 3 μl of sheep anti-human CAII antibody (Serotec), or 3 μl of goat anti-rabbit CAIV antibody (35). After washing with TBST buffer, blots were incubated for 1 h with 10 ml of TBST-M containing 1:3000 diluted donkey anti-goat IgG conjugated to horseradish peroxidase. Anti-CAII immunoblots were incubated with rabbit CAII polyclonal antibody (47) at the 35 μl/ml dilution. Blots were washed with TBST-M containing 1:3000 diluted rabbit anti-goat IgG conjugated to horseradish peroxidase. After washing with TBST buffer, blots were visualized and quantified using ECL reagent and a Kodak Image Station 440CF.

Anion Exchange Activity Assay—Anion exchange activity was monitored using a fluorescence assay described previously (41). Briefly, HEK293 cells grown on coverslips were transiently transfected. Two days post-transfection, coverslips were rinsed in serum-free DMEM and were incubated in 4 ml of serum-free media containing 2 μM BCECF-AM (37 °C for 15 min). Coverslips were then mounted in a fluorescence cuvette and were perfused alternately with Ringer’s buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM MgSO4, 2.5 mM NaHCO3, 10 mM NaHPO4, 5 mM NaNO3, 10 mM HEPES, pH 7.4) containing either 140 mM NaCl or 140 mM sodium gluconate and bubbled with air/CO2 (19:1). Fluorescence was monitored using a Photon Technologies International ICR fluorimeter at excitation wavelengths of 440 and 502.5 nm and an emission wavelength of 528.7 nm. Following calibration using the high potassium nigericin technique (47) at three pH values between 6.5 and 7.5, fluorescence ratios were converted to H+. Rates of change of pH were determined by linear regression (Kaleidagraph software) of the initial HCO3 efflux/influx and converted to rates of H+ equivalent flux across the plasma membrane according to the equation: iH+ = iH+ × pH (48), where as determined previously Rmax was 57.5 mM/mg (41). In all cases the transport activity of sham transfected cells was subtracted from the total rate to ensure that these rates consist only of the AE transport activity.

Coexpression Density Ultrasound—HEK293 cells were transiently transfected as described previously with cDNA encoding either AE1 or CAIV or co-transfected with both cDNAs. The method used to isolate glycosolphosphatidyl-enriched lipid rafts was a modified version of the Brown and Rose protocol (49). Two days post-transfection cells were incubated on ice in 2 ml of extraction buffer (140 mM NaCl, 1% (v/v) Triton X-100, 25 mM HEPES, pH 7.5) with the protease inhibitors described above for 10

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min. Samples were treated with 10 strokes in a Dounce homogenizer and centrifuged at 1,000 x g for 5 min. Then samples were made up to 4% sucrose by addition of an equal volume of 8% sucrose in extraction buffer without Triton X-100 and overlayed on 8 ml of 5–90% continuous sucrose gradients in which sucrose had also been dissolved in extraction buffer without Triton X-100. Gradients were centrifuged in a Beckman SW41 rotor at 200,000 x g for 16–24 h at 4 °C and then 1-ml fractions were removed. A sample of each fraction was prepared for SDS-PAGE sample buffer. Samples of each fraction were resolved by SDS-PAGE on 8 or 12.5% acrylamide gels and transferred to PVDF membrane. Immunoblots were probed with rabbit polyclonal antibody 1658 directed against the C terminus of human AE1, sheep anti-human CAII antibody, or goat anti-rabbit CA IV as indicated.

Rationale for 30 min. Samples were treated with 10 strokes in a Dounce homogenizer and centrifuged at 1,000 x g for 5 min. Then samples were made up to 4% sucrose by addition of an equal volume of 8% sucrose in extraction buffer without Triton X-100 and overlayed on 8 ml of 5–90% continuous sucrose gradients in which sucrose had also been dissolved in extraction buffer without Triton X-100. Gradients were centrifuged in a Beckman SW41 rotor at 200,000 x g for 16–24 h at 4 °C and then 1-ml fractions were removed. A sample of each fraction was prepared for SDS-PAGE sample buffer. Samples of each fraction were resolved by SDS-PAGE on 8 or 12.5% acrylamide gels and transferred to PVDF membrane. Immunoblots were probed with rabbit polyclonal antibody 1658 directed against the C terminus of human AE1, sheep anti-human CAII antibody, or goat anti-rabbit CA IV as indicated.

Expression of AE1, CAII, and CAIV in transfected cells. HEK293 cells were transiently co-transfected with cDNA coding for AE1, CAII, and CAIV. Two days post-transfection, cells were solubilized. Samples (5 μg of protein) were resolved by SDS-PAGE on 8 or 12.5% acrylamide gels and transferred to PVDF membrane. Immunoblots were probed with rabbit polyclonal antibody 1658 directed against the C terminus of human AE1, sheep anti-human CAII antibody, or goat anti-rabbit CA IV as indicated.

Expression of AE1, CAII, and CAIV in transfected cells. HEK293 cells were transiently co-transfected with cDNA coding for AE1, CAII, and CAIV. Two days post-transfection, cells were solubilized. Samples (5 μg of protein) were resolved by SDS-PAGE on 8 or 12.5% acrylamide gels and transferred to PVDF membrane. Immunoblots were probed with rabbit polyclonal antibody 1658 directed against the C terminus of human AE1, sheep anti-human CAII antibody, or goat anti-rabbit CA IV as indicated.

Results

Expression of AE1 and CA in HEK293 Cells—For functional assays, proteins were expressed in HEK293 cells. This cell line expresses endogenous CAII (8) yet undetectable levels of AE protein (13). All cDNAs were inserted into either the pcDNA3.1 or pRBG4(40) vector, which place them under the control of the cytomegalovirus early gene promoter. Cells were transiently co-transfected with cDNAs encoding AE1, the functionally inactive V143Y CAII, or AE1 and CAIV. Fig. 1 indicates that transient co-transfection of HEK293 cells with cDNAs encoding AE1, CAII, and CAIV results in expression of all three proteins. Cells transfected with vector alone showed no immunoreactivity with AE or CA IV antibodies but did indicate the presence of endogenous CAII at a level ~20-fold lower than in CAIIV-transfected cells (not shown). CAIV on immunoblots frequently appeared as two bands. The source of this doublet is not clear but the difference in size is consistent with either two different glycosylated forms (35) or from partial protein processing, leaving an uncleaved transmembrane anchor on the protein.

Citrate/HCO3 Exchange Activity—To measure anion exchange activity, transiently transfected cells were grown on coverslips and loaded with BCECF-AM, a pH-sensitive fluorescent dye. The coverslips were placed in a fluorescence cuvette and perfused alternately with chloride-containing and chloride-free Ringer’s buffer. In chloride-free Ringer’s buffer, chloride leaves the cell and bicarbonate enters resulting in cell alkalinization. In chloride-containing Ringer’s buffer, the opposite happens with chloride entering the cell in exchange for bicarbonate, leading to cell acidification. Following appropriate calibration using the high potassium nigericin technique (47), changes in fluorescence of BCECF provide an indirect measure of changes in intracellular pH associated with chloride bicarbonate exchange activity.

To determine the effect of CAIV on AE transport activity we co-transfected HEK293 cells individually with AE1, AE2, or AE3 and CA IV cDNAs. Co-expression of AE proteins with CA IV had no effect on the AE-mediated bicarbonate transport activity (data not shown). An effect of CA IV may not have been evident because HEK293 cells endogenously express sufficient CAII to maximize AE transport activity (8). To separate any effect CA IV might have on AE transport activity from that of CAI, we overexpressed a functionally inactive V143Y CAII mutant (38). Transfection of HEK293 cells with V143Y CAII resulted in 20-fold expression over endogenous CAII levels (not shown). V143Y CAII acts in a dominant-negative manner to displace functional wild-type CAII from cellular binding sites, thus reducing AE transport activity by blockade of the functional AE/CAII metabolon (8).

Fig. 2 shows that expression of V143Y CAII substantially reduced AE1 transport activity (53 ± 3% inhibition). Strikingly, addition of CAIV to AE1 and V143Y CAII fully rescued the transport activity of AE1, restoring the bicarbonate transport rate to the same level as cells expressing AE1 and wild-type CAII alone (Fig. 2). The CAIV-induced rescue of AE1 transport activity indicates a functional interaction between AE1 and CAIV. This result implies that the AE1 bicarbonate transport rate can be maximized by an interaction with either CAII or CAIV. To determine whether the rescue of AE1 transport activity by CAIV was dependent on the catalytic activity of CAIV, we compared the transport activity of cells expressing AE1, V143Y CAII, and CAIV before and after incubation with the CA inhibitor acetazolamide (Fig. 2). Acetazolamide is a membrane-permeant inhibitor of both CAII and CAIV that has no direct effect on anion exchange activity (50, 51). The presence of 100 μM acetazolamide abolished the CAIV-induced rescue of AE1 transport activity (50 ± 1% inhibition) (Fig. 2D). This indicates that the rescue of AE1 transport activity by CAIV was dependent on the catalytic activity of CAIV. The co-expression of V143Y CAII also reduced transport activity of AE2 and AE3 (49 ± 10 and 35 ± 1% inhibition, respectively) (Fig. 3). Fig. 3 also demonstrates that co-expression of CAIV with V143Y CAII rescued AE2 and AE3 transport activity to full capacity (85 ± 5 and 108 ± 1%, respectively), which indicates a functional interaction with CAIV.

Sucrose Density Centrifugation—CAIV resides on the extra-cellular surface of cells, anchored via a glycosylphosphatidylinositol linkage and has been localized to lipid rafts in the plasma membrane (21). Cold solubilization of membranes with Triton X-100 leaves lipid rafts intact whereas solubilizing the rest of the membrane (49). Subsequent sucrose density centrif-
Extracellular Interaction of AE1 and CAIV

FIG. 2. Effect of carbonic anhydrases on AE1 transport activity. HEK293 cells grown on coverslips were transiently co-transfected with cDNA encoding AE1 (A), AE1 and V143Y CAII (B), and AE1, V143Y CAII, and CAIV (C). Two days post-transfection, cells were loaded with BCECF-AM and placed in a fluorescence cuvette in a fluorimeter. Cells were perfused alternately with Cl−-containing (solid bar) and Cl−-free (open bar) Ringer’s buffer and fluorescence was monitored using excitation wavelengths of 440 and 502.5 nm and emission wavelength of 528.7 nm. In some experiments cells were incubated with 100 μM acetazolamide for 10 min followed by a repeat of the Ringer’s buffer perfusion in the presence of 100 μM acetazolamide. Transport activity following acetazolamide incubation was compared with that before the incubation. D, summary of transport rates expressed relative to rate for AE1 expressed alone. Error bars represent mean ± S.E. (n = 4) and asterisks represent statistical significance (p < 0.001).

FIG. 3. CAIV facilitates bicarbonate transport by AE2 and AE3. HEK293 cells were transiently transfected with cDNA encoding AE2 or AE3 and co-transfected with or without V143Y CAII and CAIV cDNA, as indicated at the bottom of the figure. Anion exchange activity was measured and rates expressed relative to the rate for AE2 (panel A) and AE3 (panel B). Error bars represent the mean ± S.E. (n = 4) and the asterisks represent statistical significance (p < 0.001).

Extracellular Interaction of AE1 and CAIV

Ultracentrifugation allows separation of proteins according to density. We used this technique to investigate the possibility of a physical interaction between CAIV and AE1. HEK293 cells transiently transfected with either AE1 or CAIV or with both AE1 and CAIV were treated with Triton X-100 and lysates were overlaid onto 5–30% continuous sucrose gradients. Following a 16–24-h ultracentrifugation, fractions were collected and the relative amount of AE1 and CAIV in each fraction was measured. Fig. 4 shows that when expressed alone, CAIV is found predominantly in fractions 3 and 4, but when AE1 is expressed alone, AE1 is found predominantly in fraction 7. However, when AE1 and CAIV are co-expressed, AE1 remains predominantly in fractions 7/8 whereas the CAIV shifts to fraction 7. The AE1-dependent shift of CAIV suggests a physical interaction between AE1 and CAIV.

CAIV Overlay Assay—The interaction between CAIV and AE was further investigated with a blot overlay assay. Cell lysates of HEK293 cells expressing one of AE1, AE2, or AE3 were resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated overnight with a cell lysate of HEK293 cells expressing CAIV. Immunoblots were then probed with anti-CAIV antibody. Fig. 5 shows that CAIV was present at positions corresponding to the migration positions of the AE proteins. No bands were observed in samples from untransfected HEK293 cells and there were no immunoreactive bands common to all lanes. Thus the bands observed represent a specific interaction of CAIV with only the AE protein present in each lane. This data suggests that there is a physical interaction between CAIV and the AE1, AE2, and AE3 anion exchange proteins.

GST Pull-down Assays of AE1 Extracellular Loops—To localize the site of AE1 interaction with CAIV, we reasoned that glycosylphosphatidylinositol-anchored CAIV must interact with the extracellular portion of AE1. The most likely candidates for a CAIV-binding site are the largest extracellular loops of AE1, EC3, and EC4 (Fig. 6). GST fusion proteins of the third and fourth extracellular loops of AE1 were used in a GST pull-down assay. GST alone, GST-AE1EC3, and GST-AE1EC4 were immobilized on glutathione-Sepharose resin and cell lysates of either sham transfected or CAIV-transfected cells were applied. After washing, proteins were eluted in SDS-PAGE sample buffer resolved on 8% polyacrylamide gels by SDS-PAGE electrophoresis, transferred to PVDF membranes, and probed for CAIV. In Fig. 7 bands were evident only in samples incubated with CAIV-containing lysates. Thus all bands observed represent a specific interaction of CAIV with only the AE protein present in each lane. This data suggests that there is a physical interaction between CAIV and the AE1, AE2, and AE3 anion exchange proteins.
levels of protein in each fraction. Immunoblots provided relative expression bars. Scanning and densitometry of immunoblots provided relative expression levels of protein in each fraction. Error bars represent mean ± S.E. (n = 3).

Fig. 4. Association of AE1 and CAIV. HEK293 cells were transiently transfected with cDNA encoding either AE1 or CAIV or co-transfected with both cDNAs, as indicated in each panel. Two days post-transfection, cells were solubilized in cold Triton X-100 and samples were overlaid on 5–30% continuous sucrose gradients. Following ultracentrifugation, 12 fractions were collected (1-top, 12-bottom) and samples of each were resolved by SDS-PAGE on 8% (AE1) or 12.5% (CAIV) polyacrylamide gels. Immunoblotted proteins were probed with either anti-AE1 (black bars) or anti-CAIV (gray bars). Scanning and densitometry of immunoblots provided relative expression levels of protein in each fraction. Error bars represent mean ± S.E. (n = 3).

Fig. 5. Blot overlay assay of CAIV on AE1, AE2, and AE3. HEK293 cells were transiently transfected individually with AE1, AE2, or AE3 cDNA. Two days post-transfection, cells were solubilized, and 5 µg of protein was resolved by SDS-PAGE on 8% acrylamide gels and transferred to PVDF membrane, as indicated in the figure. Immunoblots were blocked for 3 h with 10% TBST-M and then incubated overnight in 1% TBST-M containing a lysate of CAIV-transfected HEK 293 cells. Blots were then probed with anti-CAIV antibody. Arrows indicate the position of the AE proteins.

than did GST alone or GST-AE1EC3. This demonstrates that CAIV binds specifically to the fourth extracellular loop of AE1.

**DISCUSSION**

The data presented here show that the expression of CAIV accelerates the rate of bicarbonate transport by AE1, AE2, and AE3. HEK293 cells endogenously express CAII at a level that is sufficient to maximize the bicarbonate transport activity of the AE family (8). The effect of CAIV on AE transport was found only in the presence of V143Y CAII, which displaces endogenous CAII from its binding site in AE, greatly reducing the anion transport rate (8). Whereas bicarbonate transport by AE1, AE2, and AE3 was inhibited by 35–53% by V143Y CAII, the loss of activity was fully rescued by expression of CAIV. The rescue of AE activity by CAIV was blocked by acetazolamide, a CA inhibitor, indicating that the catalytic activity of CAIV was responsible for the rescue of the AE mediated bicarbonate transport activity.

Carbonic anhydrases and bicarbonate transport proteins are together responsible for bicarbonate metabolism and transmembrane transport. Previous studies showed that these proteins form a complex (5–7, 52, 53) and we have recently provided evidence that the physical interaction between the AE family of bicarbonate transport proteins and CAII is necessary for maximal HCO3− transport activity (8). The wide tissue distribution of CA isoforms raises the question of the possibility of the formation of a complex between bicarbonate transport proteins and other CA isoforms. In the present report we examined the relationship between the extracellular CA isoform, CAIV, and plasma membrane chloride/bicarbonate exchange proteins.

Three lines of evidence indicate that CAIV and anion exchangers form a physical complex. CAIV is localized to lipid rafts in the membrane (49). Lipid rafts are areas rich in sphingolipids and cholesterol and are known to remain intact upon cell solubilization in cold Triton X-100 (49). We compared the sedimentation of CAIV in sucrose gradients in the absence and presence of AE1. In the presence of AE1, the sedimentation of CAIV shifted from the less dense fractions where it is found when expressed alone, to the denser fractions where AE1 was localized. This result suggests that AE1 and CAIV physically interact and that AE1 pulls CAIV out of lipid rafts. In a second approach, AE1, AE2, and AE3 expressed in HEK293 cells were able to interact with CAIV from cell lysates of HEK293 cells expressing CAIV in gel overlay assays.

The third and most definitive evidence of a CAIV/AE interaction came from GST pull-down assays. As CAIV is linked to the extracellular surface of the cell, we reasoned that the AE/CAIV interaction occurred at one of the larger extracellular loops of AE1. We investigated the extracellular loops between transmembrane segments 5 and 6 (EC3) and transmembrane segments 7 and 8 (EC4). GST fusion proteins of the individual loops (GST-AE1EC3 and GST-AE1EC4) were constructed. These GST fusion proteins and control GST alone were immobilized on glutathione-Sepharose resin. Lysates prepared from HEK293 cells transfected with CAIV cDNA or sham-transfected were incubated with the GST protein-glutathione-Sepharose resin complexes. CAIV associated with the resin was detected on immunoblots. The presence of a band corresponding to the molecular weight of CAIV appeared only when lysates from CAIV-transfected cells were applied to immobilized GST-AE1EC4 (Fig. 6). This suggests that CAIV binds specifically to the fourth extracellular loop of AE1.

On the basis of these three lines of evidence we conclude that CAIV forms a complex with AE1, AE2, and AE3. The simplest explanation for our observation is that CAIV directly interacts with AE1, AE2, and AE3. We cannot rule out the possibility that another protein is required to mediate the AE/CAIV interaction. However, the requirement of an intermediary protein is highly unlikely because CAII interacts directly with AE1-AE3 (8) and any intermediary protein would have to be endogenously expressed in HEK293 cells. The increase of AE1, AE2, and AE3 bicarbonate transport activity caused by CAIV likely requires a direct interaction between CAIV and AE; localization of CAIV to the same membrane may not be sufficient to enhance bicarbonate transport rate.
The identification of EC4 as the binding site for CAIV is interesting in a number of ways. Studies of AE1 topology suggest that EC4 is the largest extracellular loop (46, 54) and therefore might be expected to form an extracellular binding site. That EC4 forms an accessible extracellular region is demonstrated by the four blood group antigens, the Wright antigen (E658K) (55), Moa (R656H), Hga (R646Q), and Swa (R646Q) (56), which are found in EC4 (Fig. 6). The Wright antigen is formed by a complex between the highly glycosylated single transmembrane protein glycophorin A and AE1 (55). Thus there is precedent for an interaction between EC4 and the extracellular moiety of an erythrocyte protein.

A study of the AE1 region from the glycosylation site at Asn642 (Fig. 6) through transmembrane segment 8 suggested that the Ser645-Leu655 region had a folded structure that was inaccessible to hydrophilic reagents, whereas the Arg656-Ile661 region had an open structure with maximum accessibility at Arg656 (46). Taken together we propose that CAIV interacts with AE1 somewhere in the Arg656-Ile661 region. Interestingly this region has been suggested to form the outer vestibule that funnels anions to and from the transport site (46). Localization of CAIV to EC4 would therefore place the enzyme as close as possible to the extracellular aspect of the anion transport site.

The structure of AE2 and AE3 differs from AE1 in that AE2 and AE3 are glycosylated on EC3 rather than EC4 and EC3 is larger than EC4 in AE2 and AE3 (57). It is therefore not clear whether AE2 and AE3 interact with CAIV in the homologous loop region or not. Nevertheless, the CAIV-mediated rescue of AE2 and AE3 bicarbonate transport activity in the presence of V143Y CAII indicates a functional interaction between CAIV and AE2 and AE3, which is also likely paralleled by a physical interaction.

We have previously characterized the first example of a transport metabolon by defining the importance of the physical and functional interaction between AE1 and CAII (8). The present study provides evidence that the extracellular-anchored enzyme CAIV is the extracellular component of the bicarbonate transport metabolon. The presence of intracellular CAII and extracellular CAIV catalytic activity in the cell and the fact that both enzymes can potentiate the bicarbonate transport activity of AE1 provides the cell with a “push-pull” mechanism for bicarbonate transport (Fig. 8).
production of HCO$_3^-$ on the one side of the membrane will provide the “push” for transport by AE and CA-mediated conversion to CO$_2$ on the other side provides the “pull” by minimization of the HCO$_3^-$ at the trans transport side. This push-pull mechanism, established by having CA catalytic activity on both sides of the plasma membrane, accelerates the AE-mediated bicarbonate transport as shown in this study.

Although the heart does not express any cytosolic CA, it expresses two extracellular CA isozymes, one of which is known to be CAIV (22). The heart also expresses AE1, AE2, and AE3 (19, 58, 59), which were all shown to require interaction with CAII for maximal transport activity to be achieved (8). Our results show that extracellular CAIV can functionally replace CAII. Therefore, despite the absence of CAII in cardiomyocytes, these bicarbonate transporters would be expected to be able to function at their maximum rate.

The kidney expresses an N-terminal truncated variant of AE1 (kAE1). Although it is generally agreed that kAE1 localizes to the basolateral surface of α-intercalated cells (60), there is also one report that kAE1 is found at the apical surface of β-intercalated cells (61). AE2 is found in the basolateral surface of many portions of the kidney (62). CAIV has been reported to be in both apical and basolateral surfaces of the proximal tubule (35, 63) and the basolateral surface of the thick ascending limb (64), but others report that CAIV is only found apically in the kidney (65). Therefore, AE1 and AE2 co-localize with CAIV in some renal cells.

CAII deficiency is an autosomal recessive condition characterized by renal tubulocalcitosis (66). Despite gross abnormalities associated with the absence of CAII, CA activity in erythrocytes is adequate and patients have sufficient CO$_2$ transport capacity (67). The findings in the present paper, along with the recent detection of CAII expression and activity in human erythrocytes (31), explain the ability of AE transport activity by CAIV in the erythrocyte to provide an explanation for the observation that CAII-deficient patients is not sufficient to prevent renal tubular acidosis in the kidney, the presence of functional CAIV in CAII-deficient patients is not sufficient to prevent renal tubular acidosis (68). Thus, despite the absence of CAII in cardiomyocytes, the results show that extracellular CAIV can functionally replace CAII.


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cytosolic CA.


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to Dr. Carol Fierke for the V143Y CAII cDNA.


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Extracellular Interaction of AE1 and CAIV

The Extracellular Component of a Transport Metabolon: EXTRACELLULAR LOOP 4 OF THE HUMAN AE1 Cl−/HCO EXCHANGER BINDS CARBONIC ANHYDRASE IV
Deborah Sterling, Bernardo V. Alvarez and Joseph R. Casey

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