A Transport Metabolon

FUNCTIONAL INTERACTION OF CARBONIC ANHYDRASE II AND CHLORIDE/BICARBONATE EXCHANGERS*

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The cytoplasmic carboxyl-terminal domain of AE1, the plasma membrane chloride/bicarbonate exchanger of erythrocytes, contains a binding site for carbonic anhydrase II (CAII). To examine the physiological role of the AE1/CAII interaction, anion exchange activity of transfected HEK293 cells was monitored by following the changes in intracellular pH associated with AE1-mediated bicarbonate transport. AE1-mediated chloride/bicarbonate exchange was reduced 50–60% by inhibition of endogenous carbonic anhydrase with acetazolamide, which indicates that CAII activity is required for full anion transport activity. AE1 mutants, unable to bind CAII, had significantly lower transport activity than wild-type AE1 (10% of wild-type activity), suggesting that a direct interaction was required. To determine the effect of displacement of endogenous wild-type CAII from its binding site on AE1, AE1-transfected HEK293 cells were co-transfected with cDNA for a functionally inactive CAII mutant, V143Y. AE1 activity was maximally inhibited 61 ± 4% in the presence of V143Y CAII. A similar effect of V143Y CAII was found for AE2 and AE3 cardiac anion exchanger isoforms. We conclude that the binding of CAII to the AE1 carboxyl-terminus potentiates anion transport activity and allows for maximal transport. The interaction of CAII with AE1 forms a transport metabolon, a membrane protein complex involved in regulation of bicarbonate metabolism and transport.

Carbon dioxide, the metabolic end product of oxidative respiration, must be effectively cleared from the human body. CO₂ diffuses out of cells into the blood stream and into erythrocytes, where it is hydrated by cytosolic carbonic anhydrase (CA). The resulting membrane-impermeant HCO₃⁻ is exported into the plasma by the plasma membrane Cl⁻/HCO₃⁻ anion exchanger (AE1), thus increasing the blood capacity for carrying CO₂. Upon returning to the lungs the process is reversed; HCO₃⁻ is transported into the erythrocyte in exchange for Cl⁻ by AE1 and dehydrated by CA, and the resulting CO₂ diffuses across the erythrocyte and alveolar membranes to be expired from the body. The 5 × 10⁴ s⁻¹ turnover rate of AE1 (1) and the high content of AE1 in the membrane (2) facilitate completion of bicarbonate transport within 50 ms during passage of an erythrocyte through a capillary (3).

AE1 is a 911-amino acid polytopic glycoprotein that facilitates the one for one electroneutral exchange of Cl⁻ for HCO₃⁻ across the plasma membrane. It consists of two major domains, a 43-kDa amino-terminal cytoplasmic domain (4), which interacts with cytoskeletal proteins and glycolytic enzymes (5), and a 55-kDa carboxyl-terminal membrane domain (4). The membrane domain spans the lipid bilayer 12–14 times (6) and is responsible for Cl⁻/HCO₃⁻ exchange activity (7). The protein terminates with a cytoplasmic 33-amino acid carboxyl-terminal domain (8, 9). A truncated form of human AE1 beginning at methionine 66 is found in kidney (10). Other plasma membrane anion exchange proteins include AE2 and AE3 and recently identified AE4, DRA (down-regulated in adenoma), and Pendrin (11–16).

In mammals 14 CA isoforms have been identified (17–19). Human erythrocytes express predominately CAI and a lesser amount of CAII (20). However, CAII accounts for the majority of carbonic anhydrase activity in human erythrocytes since it has a higher turnover rate (10⁶ s⁻¹) (20) and CAI facilitated hydration of CO₂ is inhibited by 92% in physiological concentrations of Cl⁻ (80 mM) (21). Interestingly, the erythrocyte contains ~10⁶ of the CAII isofrom (22), which is stoichiometric with AE1 copies (1.2 × 10⁶/erythrocyte) (2, 23). Effective Cl⁻/HCO₃⁻ anion exchange in erythrocytes is dependent on the activity of cytosolic carbonic anhydrase (24–27).

Several lines of evidence show an interaction between CAII and AE1. Binding of erythrocyte membranes to CAII has been shown to increase its enzymatic activity (28). This interaction is weak, however, because the bulk of carbonic anhydrase can be readily removed from isolated erythrocyte membranes (29, 30). Reaction of an anion transport inhibitor (DIDS) with AE1 altered the binding of a fluorescent inhibitor to carbonic anhydrase, suggesting a physical link between these two proteins (31). Extracellular lectin caused agglutination of AE1 and a similar redistribution of CAII on the cytosolic surface of the erythrocyte membrane (30), suggesting a physical interaction of AE1 with CAII. CAII can be co-immunoprecipitated with solubilized AE1, and finally, a sensitive microtiter assay showed that CAII but not CAI interacts with the carboxyl terminus of AE1 (30). Truncation and point mutation of the

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¶¶ The abbreviations used are: CA, carbonic anhydrase; AE, anion exchanger; BCEEFAm, 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; GST, glutathione S-transferase; GST-AE1ct, fusion of AE1 carboxyl terminus to GST; HEK, human embryonic kidney; pHᵢ, intracellular pH; TBST, Tris-buffered saline Tween; DIDS, 4,4′-diisothiocyanostilbene-disulfonate.
AE1 carboxyl terminus led to the identification of the binding site of CAII in human AE1 as LDADD (amino acids 886–890) (32). Binding assays also showed that CAII interacts with the carboxy-terminal region of AE2 (32), but interaction between AE3 and CAII has not yet been examined. The interaction between AE1 and CAII is pH-dependent (30), which suggested binding of the acidic LDADD motif of AE1 with a basic region of CAII. Truncation and mutagenesis of the basic amino-terminal region of CAII showed that it forms the AE1 binding site (33). Replacement of basic residues in the amino terminus of CAII with the equivalent residues in CAI resulted in a loss of AE1 binding (33). Truncation of the CAII amino-terminal region also resulted in loss of binding ability but did not impair enzymatic activity, implying that the function of the basic amino-terminal domain is to bind CAII to AE1 or other proteins with similar acidic binding motifs.

In this study we tested the functional consequences of the AE1/CAII interaction. Our hypothesis was that this interaction facilitates the coupling of CAII enzymatic activity and anion exchange activity, resulting in more efficient bicarbonate transport. Using HEK293 cells transiently transfected with AE1 cDNA, we determined that inhibition of endogenous CAII activity with acetazolamide resulted in a decrease of AE1 transport activity. Mutation of the AE1 LDADD acidic binding motif to LAAAA or LNANN caused a loss of CAII binding and also a decrease of AE1 transport activity. Binding of functionally inactive V143Y CAII mutant (34) had a dominant negative effect on anion transport. Overexpression of V143Y CAII also caused a reduction of AE2 and AE3 cardiac transport activity. This first demonstration of a functional interaction between CAII and AE3 cardiac leads us to conclude that binding of CAII to the carboxyl terminus of AE proteins is required for maximal transport activity. The requirement of a physical interaction between CAII and AE1 for maximal bicarbonate transport activity suggests that the AE1-CAII complex forms a functional transport metabolon, a physically associated complex of proteins in a sequential metabolic pathway (35, 36).

**EXPERIMENTAL PROCEDURES**

**Materials—**ECL chemiluminescent reagent, donkey anti-rabbit IgG conjugated to horseradish peroxidase, and Hyperfilm were from Amerham Biosciences, Inc. Poly-L-lysine and nigericin were from Sigma-Aldrich. Molecular Probes BCECF-AM was from Cederlane Laboratories Ltd. (Ontario, Canada). Glass coverslips were from Fisher. Sulfo-NHS-SIG-Biotin and streptavidin-agarose were from Pierce. Sheep anti-human carbonic anhydrase II antibody was from Serotec (Raleigh, NC). Jackson ImmunoResearch Laboratories Donkey anti-sheep conjugated to horseradish peroxidase was from BioCan Scientific (Mississauga, Canada).

**Molecular Biology—**Human AE1 cDNA (a generous gift of Drs. A. M. Garcia and H. Lodish) was inserted into the HindIII and BamHI sites of pcDNA3.1 (Invitrogen, Carlsbad, CA). AE1 was mutated in the CAII binding site (S468LDADD to LNANN and LDADD) using the CLON-TECH transformer site-directed mutagenesis kit and oligonucleotide primers from AGCT Corp. (Toronto, Canada). The mutations were confirmed using an Amersham Biosciences T7 sequencing kit. A construct for expression of wild-type human CAII was prepared by digestion of pACa containing the CAII cDNA with XbaI at the 5′ end and EcoRI at the 3′ end. The XbaI/EcoRI fragment was then cloned into XbaI/EcoRI-digested pRSBG4 vector to yield pJRC36 (37). An expression construct for the V143Y CAII mutant called pDS4 was constructed by the same strategy using V143Y cDNA supplied by Dr. Carol Fierke (34). The construct was verified by DNA sequencing performed by the Core Facility in the Department of Biochemistry, University of Alberta with an Applied Biosystems 373A DNA sequencer. Plasmid DNA for transfection was prepared using Qiagen columns (Qiagen Inc., Mississauga, Canada).

**Protein Expression—**AE proteins were expressed by transient transfection of HEK293 cells (38) using the calcium phosphate method (39). Cells were grown at 37 °C in an air/CO2 (19:1) environment in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) fetal bovine serum and 5% (v/v) calf serum.
with acetazolamide present in all buffers. Curves for transport inhibition by acetazolamide were fitted with Kaleidagraph software (Synergy Software, Reading, PA).

**Surface Processing**—Cell surface processing assays were performed as described previously (37). Briefly, HER293 cells grown in 100-mm dishes were transiently transfected with wild-type and mutant AE1 cDNA as described above. Two days post-transfection cells were washed and harvested in phosphate-buffered saline. After a wash with borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl2, 10 mM borate acid, pH 9.0), cells were incubated with 5 ml of 0.5 mg/ml Sulfo-NHS-SS-Biotin buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% (v/v) Triton X-100, 0.1% (v/v) SDS, 150 mM NaCl, 1 mM EDTA, 10 mM Tris-Cl, pH 7.5) containing protease inhibitors (0.1 mM phenylmethylsulfonil fluoride, 0.2 mM N-tosyl-l-phenylalanine, 0.1 mM N-α-tosyl-l-lysine chloromethyl ketone). The cell lysate was centrifuged for 20 min at 16,000 × g, and the supernatant was retained. Half of the supernatant was removed for subsequent SDS-PAGE analysis (total protein). Immobilized streptavidin resin (50 μl of 1–3 mg of streptavidin/ml of settled gel as a 50% slurry in phosphate-buffered saline containing 2 mM NaN3) was added to the remaining supernatant, which was then incubated overnight at 4 °C with gentle rocking. Samples were centrifuged for 2 min at 8,000 × g, and the supernatant was collected and retained for SDS-PAGE analysis (unbound fraction). The resuspended cell pellet was washed five times with immunoprecipitate buffer, and proteins were then eluted from the resin by the addition of 250 μl of SDS-PAGE sample buffer containing 1% 2-mercaptoethanol and incubation at 65 °C for 5 min. Three samples (total protein, unbound fraction, and the fraction eluted from resin) were analyzed for AE1 expression and cell surface processing by SDS-PAGE and immunoblotting as described above. Immunoblots were scanned with a Scanjet 4C flatbed scanner (Hewlett Packard, Palo Alto, CA) calibrated with a Q-14 gray-scale (Eastman Kodak Co.). Standard curves were prepared to ensure that the measurements were within the linear range of detection. Scanned images were quantified using NIH Image 1.60 software (National Institutes of Health, Bethesda, MD). The amount of protein processed to the plasma membrane was expressed as a percentage of the total.

**Binding Assay**—The binding of GST fusion proteins of the carboxyl terminus of AE1 (GST-Ct) to CAII immobilized on microtiter plates was carried out as described previously (30). Briefly, GST fusion proteins were expressed in *Escherichia coli* Bl21 and purified using glutathione-Sepharose and DEAE-Sepharose (32). Purified CAII or an inactive mutant (V143Y) (0.2–1 mg of protein/well) were chemically coupled to 96-well polystyrene microtiter plates using 1-cyclohexyl-3-(2-morpholinoethyl)carboxydimetho-p-toluenesulfonate. Plates were washed with antibody buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% (v/v) Triton X-100, 5 mM EDTA, 0.25% gelatin) and incubated with various concentrations (0–200 nM) of purified GST or GST-Ct in antibody buffer. Bound fusion proteins were detected by incubating the plates sequentially in goat anti-GST antibody, biotinylated affinity-purified anti-goat IgG, and peroxidase-labeled biotin/avidin. This was followed by incubation with the peroxidase substrate o-phenylenediamine and detection of enzymatic activity at 450 nm in a Molecular Devices ThermoMax microplate reader. Binding curves were fitted with Kaleidagraph software (Synergy Software, Reading, PA).

**Statistical Analysis**—Values are expressed ± S.E. of measurement. Statistical significance was determined using a paired *t* test with *p* < 0.05 considered significant.

**RESULTS**

**Expression of CAII and AE1 in HER293 Cells**—Functional studies were performed in transiently transfected HER293 cells. These cells express practically undetectable levels of chloride/bicarbonate exchange activity (12) but do contain endogenous carbonic anhydrase. All of the cDNAs were inserted into the pcDNA3.1 vector or pRBG4 (40), which placed them under the control of the human cytomegalovirus early gene promoter. Immunoblots of lysates from transfected HER293 cells were probed with either a polyclonal antibody directed against the carboxyl-terminal region of AE1 or an anti-CAII antibody. Fig. 1 indicates that wild-type AE1 and the LAAAA and LNANN mutants were expressed at similar levels in transiently transfected HER293 cells. Cells transfected with vector alone showed no immunoreactivity for AE1. Immunoblots using an anti-CAII antibody showed that HER293 cells express CAII and could also be transfected with cDNA for human CAII to increase the level of this enzyme. Densitometry of immunoblots indicated that CAII was overexpressed ~20-fold compared with endogenous CAII found in HER293 cells. Absolute amounts of AE1 and CAII expression were determined by immunoblot comparison of expression in transfected HER293 cells to known amounts of human erythrocyte AE1 protein and purified CAII. The stoichiometry of AE1:CAII was 1.7:1 in cells transfected with AE1 alone or 1:12 in CAII and AE1 co-transfected cells. The same amount of endogenous CAII was expressed in sham-transfected cells and cells transfected with AE1 alone.

**Effect of CAII Inhibition on Cl⁻ /HCO₃⁻ Exchange Activity**—To determine the role of CAII in facilitating AE1 transport activity, we compared the Cl⁻ /HCO₃⁻ exchange activity of cells transiently transfected with AE1 cDNA before and after incubation with acetazolamide, a sulfonamide that inhibits CA enzymatic activity without direct effect on anion exchange (25, 48). To measure AE1 transport activity, HER293 cells grown on coverslips were transiently transfected with AE1 cDNA, loaded with BCECF-AM, a pH-sensitive fluorescent dye, and mounted in a fluorescence cuvette. Cells were perfused alternately with chloride-containing (140 mM) and chloride-free Ringer’s buffer. Solutions at pH 7.4 were continually bubbled with 5% CO₂ in balanced air (PₐCO₂ = 40 mm of Hg), resulting in a constant extracellular bicarbonate concentration of 25 mM (49). In chloride-free Ringer’s buffer, Cl⁻ will leave the cell in exchange for extracellular HCO₃⁻, causing cell alkalization. The reverse happens when cells are perfused with chloride-containing buffer. BCECF fluorescence changes with pH and with appropriate calibration will give a measurement of changes in intracellular pH (pHᵢ). Previous determination of the intrinsic buffering capacity of HER293 cells (37, 40) enables the rate of change in pHᵢ to be converted to H⁺ flux. The intrinsic buffering capacity was negligible above pH 7.1; thus, H⁺ flux was determined as the product of the change in pHᵢ time and bicarbonate buffering (2.5 × [HCO₃⁻]) (47). The typical rise of intracellular pH from pH 7.2 to 7.7 in these assays corresponds to a rise in intracellular bicarbonate concentration of 35 mM. This rise occurs readily in exchange for efflux of intracellular Cl⁻, which is found in mammalian cells at 25–60 mM (50). In these assays, transport rates were determined by linear regression of the initial slopes of curves produced as pHᵢ changes. AE1 transport activity was measured, and then cells were incubated for 10 min with acetazolamide. Because acetazolamide will not covalently react with the CA, all buffers used subsequently to the incubation also contained the appropriate
concentration of acetazolamide. This allowed for comparison of anion transport activity of the same population of cells in the absence and presence of acetazolamide.

The effect of acetazolamide on anion exchange activity was monitored during both bicarbonate influx (Cl\(^{-}/\)H\(_{2}\)CO\(_{3}\)-free Ringer’s buffer) and during bicarbonate efflux (Cl\(^{-}/\)H\(_{2}\)CO\(_{3}\)-containing Ringer’s buffer). In Fig. 2A acetazolamide (100 \(\mu\)M) was initially added to transfected HEK293 cells in Cl\(^{-}/\)H\(_{2}\)CO\(_{3}\)-free Ringer’s buffer, which caused inhibition of both AE1-mediated bicarbonate influx and efflux. However, the two transport rates were not equally inhibited; bicarbonate influx was inhibited by 49 \(\pm\) 5\%, whereas bicarbonate efflux was inhibited by 62 \(\pm\) 3\%. The larger inhibition of bicarbonate efflux caused by acetazolamide was statistically significant \((p < 0.05; n = 4)\).

Fig. 2B is a dose-response curve for the effect of acetazolamide (added initially in Cl\(^{-}/\)free Ringer’s buffer) on bicarbonate efflux. AE1 bicarbonate efflux activity was maximally inhibited by 70 \(\pm\) 2\% at an acetazolamide concentration of 250 \(\mu\)M. The apparent \(K_i\) was 54 \(\mu\)M, which is higher than the 10\(^{-8}\) M value measured using purified CAII (20). The concentration of carbonic anhydrase inhibitors required to have an effect on anion exchange in erythrocytes were also found to be several orders of magnitude higher than required to inhibit CAII (24, 25). In these studies, CAII did not become rate-limiting for anion exchange until the enzyme was inhibited to greater than 99\%. This is because of the high activity of CAII relative to the transport activity of AE1 in red cells requiring doses of acetazolamide in the mM range. Acetazolamide does not directly inhibit AE transport activity because it cannot inhibit chloride/bicarbonate (48). Thus, the observed decrease in AE transport activity in the presence of acetazolamide was due to the inhibition of CA activity.

\(\text{Cl}^-/\text{HCO}_3^-\) Exchange Activity of AE1 Mutants—To determine whether the interaction of CAII with AE1 was necessary for optimal transport, the transport mediated by two different mutants of the AE1 CAII binding site (LNANN, LAAAA) was determined. Neither of these mutants was able to bind CAII (32). HEK293 cells transiently transfected with cDNA encoding wild-type AE1 (A), LAAAA mutant AE1 (B), and LNANN mutant AE1 (C) is shown.

**FIG. 2.** Full AE1 transport activity requires active carbonic anhydrase. HEK293 cells transfected with AE1 cDNA 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 440 and 502.5 nm and emission wavelength 528.7 nm. Cells were then incubated with acetazolamide for 10 min followed by a repeat of the Ringer’s buffer perfusion. Linear regression of the initial rate of change of fluorescence upon change of perfusion buffer provides a measurement of transport activity of AE1. Transport activity after acetazolamide incubation was compared with that before the incubation and expressed as a percentage of this transport activity. A, effect of 100 \(\mu\)M acetazolamide on AE1-mediated HCO\(_3^-\) transport. B, relative degree of inhibition of AE1-mediated bicarbonate efflux after acetazolamide incubation. S.E. bars are indicated \((n = 4)\).

**FIG. 3.** Transport activity of AE1 mutant proteins unable to bind CAII. HEK293 cells transiently transfected with AE1 cDNA only were loaded with BCECF-AM. Cells were perfused alternately with Cl\(^{-}\)-containing (solid bar) and Cl\(^{-}\)-free (open bar) Ringer’s buffer, and fluorescence was monitored using excitation wavelengths 440 and 502.5 nm and emission wavelength 528.7 nm. Transport activity of HEK293 cells transfected with wild-type AE1 (A), LAAAA mutant AE1 (B), and LNANN mutant AE1 (C) is shown.
assays. Wild-type AE1 had a transport rate of about 10 times that of the mutant LAAAA and LNANN AE1 proteins (40 ± 0.6 versus 4 ± 0.6 and 3 ± 0.6 mM H⁺ equivalent/min, respectively). The two mutants of the AE1 carboxyl-terminal tail, thus, clearly had reduced transport rates relative to wild type.

The large difference in transport activity between the wild-type and mutant AE proteins may be due to differences in protein expression at the plasma membrane. The total amount of expression of wild-type AE1 and the two mutants was similar (Fig. 1). The activity assay used to measure transport rate only measures the activity of protein expressed at the plasma membrane; thus, any protein retained intracellularly will be observed as nonfunctional. To address the possibility that introducing mutations into the protein interferes with the ability of the protein to be properly processed to the plasma membrane, we investigated the amount of expressed protein present in the plasma membrane for each of the AE transport proteins investigated by cell surface biotinylation. The fraction of protein expressed at the plasma membrane was similar for wild-type and LNANN mutants (32 ± 2 and 37 ± 3%, respectively; n = 5) and statistically higher for the LAAAA mutant (61 ± 3%) than for wild-type AE1 (p < 0.0002, n = 5). Thus, the lower activity of the two carboxyl-terminal mutants of AE1 is not explained by reduced expression or processing to the cell surface.

Overexpression of CAII in HEK293 Cells—Because the stoichiometry of AE1:CAII in AE1-transfected HEK293 cells was 1.7:1, we determined whether CAII activity was rate-limiting to measured AE transport activity. CAII was co-expressed with the AE proteins by transient transfection, and transport activity was also determined. Fig. 4 summarizes the transport activity of the AE proteins when expressed alone or along with excess CAII. Co-transfection with wild-type CAII cDNA had no significant effect on transport activity of any of the AE1 constructs, confirming that the endogenous amount of CAII present in HEK293 is not rate-limiting to AE transport activity. In addition overexpression of CAII was not able to rescue the binding defect of the LAAA or LNANN mutants.

Effect of CAII V143Y on AE1 Cl⁻/HCO₃⁻ Exchange Activity—The above experiments suggest that binding of CAII to the carboxyl terminus of AE1 is required for full AE1 activity. Another experiment to test this hypothesis was to co-express a functionally inactive CAII mutant (34) with AE proteins. Overexpression of the CAII V143Y mutant should compete with the endogenous wild-type CAII for binding to the AE1 carboxyl terminus. To assess the relative binding affinities of CAII and V143Y CAII for the AE1 carboxyl terminus, CAII proteins were immobilized on microtiter dishes and incubated with varied concentrations of GST-AE1ct (squares) and GST alone (circles). Bound proteins were detected by incubation of plates sequentially with goat anti-GST antibody, biotinylated rabbit anti-goat IgG, and then peroxidase-labeled biotin/avidin. This was followed by incubation with substrate o-phenyldiamine and detection of enzymatic activity at 450 nm in a microplate reader. Quadruplicate measurements for each concentration of protein were made on the same plate. Error bars represent S.E. of the mean (n = 4).
Anion exchange proteins and CAII are together responsible for bicarbonate transport and metabolism. Chloride/bicarbonate anion exchange in erythrocytes is dependent for its full effectiveness on CAII activity (24–27). CAII provides the substrate for bicarbonate efflux by AE1 and converts the bicarbonate that enters via AE1 to CO₂ and H₂O. Early studies suggest that AE1 and CAII form a complex (31). The CAII binding site on AE1 was recently identified as the acidic motif LDADD in the carboxyl-terminal tail of AE1 (30, 32). However the effect of binding CAII to the carboxyl terminus of AE1 upon transport activity was unknown. In the present report we have examined the relationship between CAII functional activity and plasma membrane chloride/bicarbonate exchange activity. Our data showed that inhibition of CAII by acetazolamide maximally impaired AE1 transport activity by 70 ± 2%, indicating that CAII activity was required for optimal chloride/bicarbonate exchange activity. Two mutants of AE1 shown previously to be unable to bind CAII (32) had anion exchange activity barely above background (10% of wild-type AE1 activity). The defect in transport activity of these mutants was shown not to be due to reduced expression or processing to the cell surface. Taken together these data suggest that direct interaction with CAII is required for full anion exchange activity by AE1.

The most definitive experiment leading to the above conclusion was the co-expression in HEK293 cells of the functionally inactive V143Y CAII mutant along with AE1 in the presence of endogenous CAII. Data presented here showed that wild-type CAII and the V143Y mutant bind the AE1 carboxyl terminus with equal affinity (Fig. 5). We found that increasing levels of V143Y CAII expression proportionately inhibited AE1 transport activity to a maximum level of 60% inhibition. The level of inhibition is similar to the effect of inhibition of CAII by acetazolamide on anion transport activity. This result would be observed if CAII expression reduced the level of AE1 expression. However, overexpression of wild-type CAII did not affect AE1 activity. Also, when the level of AE1 expression was measured on immunoblots, the level of AE1 expression was constant at all levels of V143Y CAII expression. We therefore interpret the data from the V143Y experiment as follows. HEK293 cells transfected with CAII cDNA overexpress the protein about 20 times the level of the endogenous protein. Thus, the introduced CAII effectively will displace endogenous CAII from its binding site on AE1. In the case of cells transfected with wild-type CAII, this has no effect on AE1 activity because the level of CAII is not rate-limiting to transport, and introduced CAII is as catalytically active as the endogenous CAII. However, when V143Y CAII is overexpressed, it will displace the endogenous wild-type CAII from its binding site on AE1. Under these conditions we observed a substantial decrease in AE1 transport activity. Because the total amount of functional CAII activity is constant between cells transfected with V143Y CAII and cells transfected with AE1 alone, we conclude that the decrease of AE1 transport activity is due to displacement of CAII from the carboxyl-terminal tail of AE1. A similar dominant negative effect of V143Y CAII was found for transport activity by the AE2 and AE3 cardiac anion exchange proteins. We conclude that binding of CAII to the carboxyl-terminal tail of AE1 is essential for maximal AE transport activity. This finding may generalize to all bicarbonate transporters since CAII binding to both AE2 and AE3 cardiac anion exchange proteins is required for full function of these proteins as well. Modulation of the interaction of CAII with anion exchangers would provide a powerful mechanism to regulate bicarbonate transport.

The stoichiometry of AE1 and CAII expression in transfected cells is informative. We found that in AE1-transfected cells the...
Fig. 7. Alignment of amino acid sequences of putative cytoplasmic, carboxyl termini of bicarbonate transport proteins. Potential CAII binding sites (underlined) consisting of a hydrophobic residue followed by a short cluster containing 2–3 acidic residues are indicated. The first letter of each sequence name refers to the species. h, human; r, rabbit. AE1 (60); AE2 (61); AE3 (51); AE4a (13); NBC1b (62); NBC3 (63); NDCBE1 (64); Pendrin (65); DRA, (GenBank™ accession number XM004892). Cytoplasmic tail sequences were identified by homology with AE1. However, the sequences of DRA and Pendrin were sufficiently different from the other proteins that the cytoplasmic tail sequences could not be confidently identified. Sequences shown represent carboxyl-terminal hydrophilic regions.

Fig. 8. Effect of CAII V143Y on AE2 and AE3 cardiac transport activity. HEK293 cells were transiently co-transfected with AE2 or AE3 cardiac cDNA (3.8 μg) and 3.8 μg of either wild-type or V143Y CAII cDNA, and anion exchange activity was measured. A, relative transport activity of cells transiently transfected with AE2 cDNA alone and with CAII isoforms as indicated. B, relative transport activity of cells transiently transfected with AE3 cDNA alone and with CAII isoforms as indicated. S.E. bars are also indicated (n = 4), and asterisks denote a significant difference in transport rates (p < 0.05) relative to transport rate of AE protein expressed alone.

total AE1:endogenous CAII stoichiometry was 1:7.1, whereas co-transfection of HEK293 cells with AE1 and CAII caused the stoichiometry to change to 1:12. Because our data show that only 32% of AE1 is processed to the cell surface, the assumption that all CAII is localized to the plasma membrane implies a significant difference in transport rates (p < 0.05) relative to transport rate of AE protein expressed alone.

AE1-transfected HEK293 cells and erythrocytes, anion transport activity is rate-limiting to the transmembrane bicarbonate flux. The assay used to assess bicarbonate transport rate in transfected HEK293 cells uses the rate of change of intracellular pH induced by transmembrane bicarbonate transport as a measure of bicarbonate flux. This is valid since the rate of CO₂/bicarbonate conversion is much faster than the rate of bicarbonate transport.

Three different methods were used to alter carboxic anhydrase activity or localization in HEK293 cells; they are inhibition with acetazolamide, mutation of the AE1 carboxyl-terminal region, and overexpression of V143Y CAII. Each of these methods inhibited AE1 transport activity to differing extents; 90% inhibition was seen in the AE1 mutants, whereas 60–70% inhibition was induced by acetazolamide or the V143Y CAII dominant-negative mutant. The larger effect seen by mutation of the AE1 carboxyl terminus may reflect the complete abolition of CAII binding caused by the mutation. However, we cannot rule out the possibility that these mutants may be functionally compromised in some way unrelated to CAII binding.

At 250 μM acetazolamide, maximal reduction of observed transport activity was 70%, and the Kᵢ for the effect of acetazolamide on observed AE1 activity was 54 μM, much higher than the Kᵢ (10 μM) for the effect of acetazolamide on CAII activity (20). This observation may be due to the fact that endogenous carboxic anhydrase activity of AE1-transfected HEK293 cells is much higher than the total anion exchange activity. Thus, no reduction of anion exchange activity measured in our assay would be expected until CAII was nearly completely inhibited, as found in the red cell system (24, 48).

Overexpression of V143Y CAII may not inhibit anion exchange as potently as mutation of the AE1 carboxyl terminus for two reasons. V143Y CAII has 3000-fold lower catalytic activity than wild-type CAII (34). Although this is a great reduction, the catalytic rate of AE1 is reduced by acetazolamide or the V143Y CAII mutant, this likely accounts for the residual AE1 activity observed.

Data presented here supports the idea that the complex of AE1 and CAII forms a bicarbonate transport metabolon (30, 32, 52). A metabolon is a complex of enzymes involved in a linked metabolic pathway that allows metabolites to move easily from one active site to the next (35, 36), a process also called channeling. Known enzymatic metabolons include the enzymes of...
the glycolysis, citric acid, and urea cycles. Channeling allows for increased flux through the pathway as it limits the loss of intermediates by diffusion. In addition, a metabolon would allow the creation of specific pools of substrates. Because AE1 can transport a range of small anions, localization of CAII effectively concentrates bicarbonate at the transport site, favoring its transport over other substrates in the cell. The presence of similar numbers of AE1 and CAII copies in the erythrocyte (2, 22, 23) provided the initial support for the theory of a metabolon complex between AE1 and CAII. Our observation of decreased transport activity upon disruption of the CAII/AE1 interaction by mutagenesis, or overexpression of V143Y CAII provides functional evidence for a bicarbonate transport metabolon. Fig. 9 is a model that illustrates acceleration of bicarbonate transport by a bicarbonate transport metabolon. The model illustrates that localization of CAII to the AE1 carboxy terminus maximizes the local concentration of bicarbonate at the transport site of AE1 during bicarbonate efflux. Conversely, during bicarbonate influx CAII minimizes the local concentration of bicarbonate, by conversion to carbon dioxide. Thus, the binding of CAII to AE1 localizes the enzyme to the cytosolic surface of the membrane where it can facilitate CO2 movement across the lipid bilayer.

We compared the Cl⁻/HCO₃⁻ exchange activity of AE1-transfected cells before and after incubation with acetazolamide. In anion exchange assays cells were alternately perfused with Cl⁻–free and Cl⁻–containing Ringer’s buffer. Thus, AE1 transported Cl⁻ out of or into the cell down a concentration gradient. In exchange, bicarbonate was transported into or out of the cell in a coupled one for one exchange process. Acetazolamide (100 μM) inhibited AE1-mediated bicarbonate influx by 49 ± 5 and efflux by 62 ± 3%, which was a statistically significant difference. We have shown that maximal AE activity is observed when CAII directly associates with the AE1 carboxyl terminus. As illustrated by Fig. 9, the difference in acetazolamide effect on bicarbonate efflux rate versus influx rate reflects the fact that during bicarbonate efflux, diminished transport will occur in the absence of CAII because CAII produces the bicarbonate substrate for efflux. The transport rate directly depends on the local bicarbonate concentration produced by CAII. During bicarbonate influx, bicarbonate is present at 25 mM on the extracellular face irrespective of CAII. The acceleration of transport observed in the presence of CAII is due to the consumption of influxed bicarbonate. Although this accelerates bicarbonate transport, the effect is not quite as large as CAII action is during efflux.

Several other bicarbonate transport proteins have recently been identified including AE4, DRA, Pendrin, and sodium bicarbonate co-transporters (NBC). AE4 was recently identified in apical membranes of β-intercalated cells in the kidney and functionally characterized (13). Cl⁻/HCO₃⁻ exchange function in the human colon and ileum has been attributed to the protein DRA (14, 15). Pendrin is expressed in the thyroid gland (54), ear (55), and kidney (16). Expression in HEK293 cells demonstrated that Pendrin can function as a Cl⁻/OH⁻, Cl⁻ / HCO₃⁻, and Cl⁻/formate exchanger (16). Sodium/bicarbonate co-transporters have been characterized in the kidney (56, 57). Sequence alignment of the extreme carboxyl-terminal tail of bicarbonate transport proteins (Fig. 7) indicates that some of these proteins also have potential CAII binding sites. It is therefore possible that interaction with CAII maximizes transport activity of many or all bicarbonate transporters.

We have presented evidence for a transport metabolon linking a cytosolic enzyme to a transporter. This physical interaction facilitates the movement of substrate from one through the other. Our study clearly demonstrates that AE1, AE2, and AE3 all require CAII binding at their carboxyl terminus for maximal bicarbonate transport activity; sequence alignments suggest interaction with CAII could be important for a host of bicarbonate transporters. The large effect of CAII binding on transport activity also suggests an effective mechanism for regulation of bicarbonate transport. Modulation of the CAII/transport protein interaction, for example by phosphorylation, would profoundly influence the rate of bicarbonate transport. Interestingly, hypertonic treatment of human erythrocytes causes phosphorylation of Tyr-904, adjacent to the CAII binding site (58, 59). Recruitment of CAII to the carboxyl-terminal tail of anion exchangers could provide a mechanism to increase chloride/bicarbonate exchange activity.

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REFERENCES

1. Jennings, M. L. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 397–430
2. Steck, T. L., Fairbanks, G., and Wallach, D. F. (1971) Biochemistry 10, 2617–2624
3. Lodish, H., Berk, A., Zipursky, S., Matsudaira, P., Baltimore, D., and Darnell, J. (2000) Molecular Cell Biology, p. 606, W. H. Freeman and Co., New York
4. Steck, T. L., Ramsay, B., and Strapazon, E. (1976) Biochemistry 15, 1153–1161
5. Low, P. S. (1986) Biochem. Biophys. Res. Commun. 145, 145–167
6. Tannen, M. J. (1997) Mol. Membr. Biol. 14, 155–165
7. Grinstein, S., Ship, S., and Rothstein, A. (1978) Biochim. Biophys. Acta 507, 294–304
8. Wainwright, S. D., Tannen, M. J., Martin, G. E., Yendle, J. E., and Holmes, C. (1989) Biochem. J. 258, 211–220
9. Lieberman, D. M., and Reithmeier, R. A. F. (1988) J. Biol. Chem. 263, 10022–10028
10. Kollett-Jona, A., Wagner, S., Huener, S., Appelhans, H., and Drenckhahn, D. (1993) Am. J. Physiol. 262, F813–F821
11. Alper, S. L., Kupito, R. R., Libresco, S. M., and Lodish, H. F. (1988) J. Biol. Chem. 263, 17092–17099
12. Kupito, R. R., Lee, B. S., Simmons, D. M., Lindsey, A. E., Morgans, C. W., and Schneider, K. (1989) Cell 59, 927–937
13. Tsuganezawa, H., Koba, K., Iyori, M., Araki, T., Kusum, A., Watanabe, S., I., Kaneko, A., Fukao, T., Monka, T., Yoshida, T., Kim, D. K., Kanai, Y., Endou, H., Hayashi, M., and Saruta, T. (2001) J. Biol. Chem. 276, 8180–8189
14. Rajendran, V. M., Black, J., Arditto, T. A., Sangan, P., Alper, S. L., Schweins, C., Kusugam, M., and Binder, H. J. (2000) Am. J. Physiol. Gastrointest. Liver Physiol. 270, 931–942
15. Melvin, J. E., Park, K., Richardson, L., Schultheiss, P. J., and Shull, G. E. (1999) J. Biol. Chem. 274, 22655–22661
16. Soleiman, M., Greeley, T., Petrovic, S., Wang, Z., Amlal, H., Kopp, P., and...
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Burnham, C. E. (2001) *Am. J. Physiol. Renal Physiol.* **280**, 356–364
17. Mori, K., Ogawa, Y., Eihara, K., Tamura, N., Tashiro, K., Kuwahara, T., Mukoyama, M., Sugawara, A., Ozaki, S., Tanaka, I., and Nakae, K. (1999) *J. Biol. Chem.* **274**, 15701–15705
18. Kivelä, A., Parkkila, S., Saarnio, J., Karttunen, T. J., Kivelä, J., Parkkila, A. K., Waheed, A., Sly, W. S., Grubb, J. H., Shah, G., Tureci, O., and Rajaniemi, H. (2000) *Am. J. Pathol.* **156**, 577–584
19. Tureci, O., Sahin, U., Vollmar, E., Siemer, S., Gottert, E., Seitz, G., Parkkila, A. K., Shah, G. N., Grubb, J. H., Fleureschuh, M., and Sly, W. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7608–7613
20. Maren, T. H. (1967) *Physiol. Rev.* **47**, 595–781
21. Maren, T. H., Hayburn, C. S., and Liddell, N. E. (1976) *Science* **191**, 469–472
22. Tashian, R. E., and Carter, N. D. (1976) *Adv. Hum. Genet.* 7, 1–56
23. Ship, S., Shami, Y., Breuer, W., and Rothstein, A. (1977) *J. Membr. Biol.* **33**, 311–323
24. Maren, T. H., and Wiley, C. W. (1970) *Med. Pharmacol.* 6, 439–440
25. Cousin, J. L., Motais, R., and Solà, P. (1975) *J. Physiol. (Lond.*) **233**, 385–399
26. Jacobs, M. H., and Stewart, D. R. (1942) *J. Gen. Physiol.* **25**, 539–552
27. Keilen, D., and Mann, T. (1941) *Nature* **148**, 493–496
28. Parkes, J. L., and Coleman, P. S. (1989) *Arch. Biochem. Biophys.* **275**, 459–468
29. Randall, R. F., and Maren, T. H. (1972) *Biochim. Biophys. Acta* **268**, 730–732
30. Vince, J. W., and Reithmeier, R. A. F. (1998) *J. Biol. Chem.* **273**, 28430–28437
31. Kifor, G., Toon, M. R., Janoshazi, A., and Solomon, A. K. (1995) *J. Membr. Biol.* **134**, 169–179
32. Vince, J. W., and Reithmeier, R. A. F. (2000) *Biochemistry* **39**, 5527–5533
33. Vince, J. W., Karlsson, U., and Reithmeier, R. A. F. (2000) *Biochemistry* **39**, 13344–13349
34. Fierke, C. A., Calderone, T. L., and Krebs, J. F. (1991) *Biochemistry* **30**, 11054–11060
35. Sere, P. A. (1985) *Trends Biochem. Sci.* **10**, 109–110
36. Sere, P. A. (1987) *Annu. Rev. Biochem.* **56**, 89–124
37. Sterling, D., and Casey, J. R. (1999) *Biochem. J.* **344**, 221–229
38. Golam, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) *J. Gen. Virol.* **52**, 59–72
39. Ruetz, S., Lindsey, A. E., Ward, C. L., and Kopito, R. R. (1993) *J. Biol. Chem.* **268**, 11448–11454
40. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
41. Laemmli, U. K. (1970) *Nature* **227**, 680–685
42. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
43. Tang, X. B., Fujinaga, J., Kopito, R., and Casey, J. R. (1998) *J. Biol. Chem.* **273**, 22545–22553
44. Casey, J. R., Lieberman, D. M., and Reithmeier, R. A. F. (1989) *Methods Enzymol.* **173**, 494–512
45. Thomas, J. A., Bachhau, R. N., Zinniak, A., and Raeker, E. (1979) *Biochemistry* **18**, 2210–2218
46. Roos, A., and Boron, W. F. (1981) *Physiol. Rev.* **61**, 296–434
47. Cousin, J. L., and Motais, R. (1976) *J. Physiol.* **256**, 61–80
48. Zeidel, M. L., Silva, P., and Seifer, J. L. (1986) *J. Clin. Invest.* **77**, 1682–1688
49. Pilas, B., and Durack, G. (1997) *Cytometry* **28**, 316–322
50. Yannoukos, D., Stuart-Tilley, A., Fernandez, H. A., Fey, P., Duyk, G., and Alper, S. L. (1994) *Circ. Res.* **75**, 603–614
51. Reithmeier, R. A. F. (2001) *Blood Cells Mol. Dis.* **27**, 35–39
52. Miles, E. W., Rhee, S., and Davies, D. R. (1999) *J. Biol. Chem.* **274**, 12193–12196
53. Scott, D. A., Wang, R., Kreman, T. M., Sheffield, V. C., and Karniski, L. P. (1999) *Nat. Genet.* **21**, 440–443
54. Everett, L. A., Morsli, H., Wu, D. K., and Green, E. D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9727–9732
55. Boron, W. F., and Boulaipa, P. L. (1989) *Kidney Int.* **36**, 392–402
56. Burnham, C. E., Amlal, H., Wang, Y., Shull, G. E., and Soleimani, M. (1997) *J. Biol. Chem.* **272**, 19111–19114
57. Minetti, G., Seppi, C., Ciana, A., Baldini, C., Low, P. S., and Bravelli, A. (1998) *Biochem. J.* **335**, 305–311
58. Yannoukos, D., Meyer, H. E., Vasseur, C., Drianceur, C., Wajcem, H., and Bursaux, E. (1991) *Biochim. Biophys. Acta* **1068**, 70–76
59. Tannen, M. J., Martin, P. G., and High, S. (1988) *Biochem. J.* **256**, 703–712
60. Gehrig, H., Muller, W., and Appelhans, H. (1992) *Biochim. Biophys. Acta* **1130**, 326–328
61. Choi, I., Romero, M. F., Khandouzi, N., Bril, A., and Boron, W. F. (1999) *Am. J. Physiol.* **276**, C576–C584
62. Pushkin, A., Abuladze, N., Lee, I., Newman, D., Hwang, J., and Kurtz, I. (1999) *J. Biol. Chem.* **274**, 16569–16575
63. Grich�chenko, I. I., Choi, I., Zhang, X., Bray-Ward, P., Russell, J. M., and Boron, W. F. (2001) *J. Biol. Chem.* **276**, 8358–8363
64. Baldwin, C. T., Weiss, S., Farrer, L. A., De Stefano, A. L., Adair, R., Franklyn, B., Kidd, K. K., Korostinshesky, M., and Bonne-Tamir, B. (1995) *Hum. Mol. Genet.* **4**, 1637–1642