Evidence against a Direct Interaction between Intracellular Carbonic Anhydrase II and Pure C-terminal Domains of SLC4 Bicarbonate Transporters*

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Based on solid-phase binding assays with enzyme-linked immunosorbent assay detection, previous investigators suggested that intracellular carbonic anhydrase II (CA II) interacts at high affinity with the C-terminal (Ct) domains of SLC4 bicarbonate-transport proteins, expressed as glutathione S-transferase (GST) fusion proteins, to form functional HCO₃⁻ metastons. Here we re-evaluated this protein-protein interaction using two solid-phase binding assays. We first compared the ability of the Ct domain of three SLC4 transporters, SLC4-A1 (AE1), SLC4-A4 (NBCe1), and SLC4-A8 (NDCBE), to bind immobilized CA II, using enzyme-linked immunosorbent assay detection. We found that when expressed as GST fusion proteins, all three bind to CA II (Kₐ 300–600 nM) better than does pure GST. However, we detected no binding of pure SLC4-Ct peptides to immobilized CA II. Second, we reversed assay orientation by immobilizing the SLC4-Ct fusion proteins or peptides. We found that more CA II binds to GST than to any of the three GST-SLC4-Ct fusion proteins. Furthermore, we detected no binding of CA II to any of the immobilized pure SLC4-Ct peptides. Finally, we used surface plasmon resonance to detect possible rapid interactions between CA II and the pure peptides. Although we detected acetazolamide binding to immobilized CA II and specific antibodies binding to immobilized SLC4-Ct peptides, we detected no binding of CA II to immobilized SLC4-Ct or vice versa. Thus, although an HCO₃⁻ metabolon may exist, CA II cannot bind directly to pure SLC4-Ct peptides and can bind to GST-SLC4-Ct fusion proteins only when the CA II is immobilized and the fusion protein is soluble, and not vice versa.

The transport of acid and base equivalents across cell membranes is important for virtually all cells and, in addition, is critical for the physiology of certain epithelia. One of the most important families of acid-base transporters is the solute carrier 4 (SLC4) family of HCO₃⁻ (or, perhaps in some cases, CO₂⁻⁻) transport proteins. In humans, this family is composed of 10 members, among which are the proteins that perform Na⁺-independent Cl⁻-HCO₃⁻ exchange (e.g. AE1–3), Na⁺-coupled anion exchange (e.g. NDCBE), and electroneutral (e.g. NBCn1) and electrogenic (e.g. NBCe1 and NBCe2) Na/HCO₃⁻ cotransport (1). These proteins are important for the regulation of intracellular pH (pHₗ) and play crucial roles in the epithelial absorption of HCO₃⁻ (e.g. in the renal proximal tubule) and secretion of HCO₃⁻ (e.g. in the pancreatic duct).

All SLC4 proteins are hypothesized to share a similar topology in the cell membrane, i.e. these proteins have relatively long cytoplasmic N-terminal domains composed of a few hundred to several hundred residues, followed by 10–14 transmembrane (TM) domains (connected by endo- and exofacial loops), and end with relatively short cytoplasmic C-terminal (Ct) domains composed of ~30 to ~90 residues. Although the Ct domain comprises a small percentage of the size of the protein, this domain in some cases (i) has PSD-95/Discs Large/ZO-1-binding motifs (2, 3) that may be important for protein-protein interactions (e.g. AE1, AE2, and NBCn1), (ii) is important for trafficking to the cell membrane (e.g. AE1 and NBCe1 (4, 5)), and (iii) may provide sites for regulation of transporter function via protein kinase A phosphorylation (e.g. NBCe1 (6)).

Recently, several studies (7–13) have reported that the Ct domain of several SLC4 members (SLC4-Ct) may directly associate with carbonic anhydrase II (CA II). It has been hypothesized that this complex forms an HCO₃⁻ transport metabolon, in which CA II enhances the function of an SLC4 transporter by directly providing it with substrate (i.e. HCO₃⁻). However, a recent study from our laboratory indicates that CA II does not increase the electrical current carried by NBCe1 (14).

The primary tool that others have used to study the interaction between CA II and the Ct domains of SLC4 proteins is a solid-phase binding assay involving enzyme-linked immunosorbent assay (ELISA) detection (7–9, 13), i.e. peptides representing the Ct domains of human AE1, AE2, and NBCn1, expressed as recombinant fusion proteins with trematode (Schistosoma japonicum) glutathione S-transferase (GST), bind to immobilized CA II, as measured by an ELISA for GST (7, 8, 14).
CA II and SLC4 C-terminal Domains

A. Control GST

GST-I EGRGI

B. GST-AE1-Ct

GST-I EGRGI RHNVELQLDADDAKTFDEEEGRDEYDEVAMPV

C. GST-NBCE1-Ct

GST-I EGRGI SQHDL SFLDDVP EKDK KKEDEKKKKGSLSDNDD DCPYSEK P SIK IPDIMEQ Q PFLSDKPSDRERSTPF LEHRH TSC

D. GST-NDCBE-Ct

GST-I EGRGI SKRELSWLDLMPESK KKLDDAKKEEVEIVLAPT VYLGASNYRT

E. AE1-Ct

GP RNVELQ LDADDAKTFDEEEGRDEYDEVAMPV

F. NBCE1-Ct

GP SQHDL SFLDDVP EKDK KKEDEKKKKGSLSDNDD DCPYSEK P SIK IPDIMEQ Q PFLSDKPSDRERSTPF LEHRH TSC

G. NDCBE-Ct

GP SKRELSWLDLMPESK KKLDDAKKEEVEIVLAPT VYLGASNYRT

13). Supporting evidence also includes recombinant NBCE1-Ct peptides that, under nonreducing conditions, are reported to interact with the following: (i) soluble CA II, as measured by isothermal titration calorimetry (10), and (ii) an immobilized GST-CA II fusion protein, as measured by pulldown assays (11).

Truncation and/or mutagenesis experiments suggest that a CA II-binding motif in the SLC4-Ct domains is composed of a hydrophobic leucine residue followed by at least two acidic residues (shown in boldface) in the next four (e.g. 886LDADD in AE1). The proposed CA II-binding motif is found in the proximal portion of the Ct domain (≈10–14 residues after the last TM domain) and is conserved among most SLC4 transporters. In the Ct domains of some SLC4 members (e.g. NBCE1, NBCn1, and NDCBE), a more distal, putative CA II-binding motif also exists (see Fig. 1). However, data on the importance of this second motif are conflicting (see Refs. 11 and 13). As far as the target site on CA II is concerned, truncation and mutagenesis studies (9) suggest that the aspartate residues of the AE1-Ct (886LDADD) interact ionically with positively charged residues (His and Lys) within the first 17 amino acids of CA II.

The affinity of SLC4-Ct proteins for CA II remains unclear. The initial estimates of the apparent affinity of GST-AE1-Ct for immobilized CA II were half-maximal binding ($K_{1/2}$) of 20 nM (7). However, a later study showed that a synthetic AE1-Ct peptide, with Cys replaced by Ala, prevented binding of GST-AE1-Ct to immobilized CA II with a $K_{1/2}$ of 100 μM (9), and a further analysis suggested that the true affinity could be as high as 300 μM (15). The authors hypothesized that this apparent discrepancy between the $K_{1/2}$ values of GST-AE1-Ct and pure AE1-Ct peptides occurs because (i) the pure peptide is not sufficiently well structured to interact stably with CA II, and/or (ii) GST dimerization enhances the avidity of the interaction of GST-AE1-Ct with CA II by a “two-point attachment” (8). On the other hand, a soluble pure NBCE1-Ct peptide is reported to interact with soluble CA II (assayed by isothermal titration calorimetry) with a $K_d$ of 160 nM (10). Furthermore, the aforementioned pulldown assays with pure NBCE1-Ct peptides and immobilized GST-CA II are consistent with a high affinity interaction (11).

The goal of this study was to extend the previous work of others by examining the binding of pure SLC4-Ct peptides (rather than GST fusion proteins) to immobilized CA II and vice versa. Our initial approach was to use the solid-phase binding assay with ELISA detection. First, we compare the ability of the Ct of three SLC4 members, SLC4-A1 (AE1), SLC4-A4 (NBCE1), and SLC4-A8 (NDCBE), to bind to immobilized CA II.

In the Ct domains of some SLC4 members (e.g. NBCE1, NBCn1, and NDCBE), a more distal, putative CA II-binding motif also exists (see Fig. 1). However, data on the importance of this second motif are conflicting (see Refs. 11 and 13). As far as the target site on CA II is concerned, truncation and mutagenesis studies (9) suggest that the aspartate residues of the AE1-Ct (886LDADD) interact ionically with positively charged residues (His and Lys) within the first 17 amino acids of CA II.

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SLC4-Ct (i.e. after removing GST), we are unable to detect binding of any of the three SLC4-Ct constructs to immobilized CA II. Second, we repeat the first two sets of studies after reversing the orientation of the proteins to the more physiological one in which the SLC4-Ct is immobilized and the CA II is soluble. We find that soluble CA II fails to bind to immobilized SLC4-Ct domains, whether or not they are fused to GST. Finally, we use surface plasmon resonance (SPR) to detect possible rapid interactions. Regardless of whether CA II or an SLC4-Ct peptide is immobilized, we detect no interaction between CA II and any of the peptides. We conclude that pure SLC4-Ct domains do not directly bind to CA II. Although we cannot rule out the existence of an HCO₃ metabolon, if an interaction between CA II and any of the SLC4-Ct domains occurs in vivo, at least one of the following must be true: 1) the intact SLC4 protein promotes a unique structure on the SLC4-Ct, enabling it to bind CA II; 2) an intermediary protein mediates the interaction; or 3) the CA II binds to another part of the SLC4 protein.

EXPERIMENTAL PROCEDURES

Generation and Purification of Recombinant Human CA II

The human CA II used in this study was identical to that used by our laboratory in a previous study (14). In brief, we expressed a pet31F1 vector, modified to code for human CA II (generously provided by Dr. David N. Silverman, University of Florida), in Rosetta 2 BL21 Escherichia coli (Novagen, Madison, WI), using an approach similar to that described by others (16). To isolate and purify CA II from the E. coli, we used a p-aminomethylbenzenesulfonamide-linked agarose resin (Sigma) and followed a protocol similar to that of Whitney (17). We further purified CA II by gel filtration chromatography (AKTA FPLC; GE Healthcare) using a Superdex 75 HR 16/60 column (GE Healthcare) equilibrated in FPLC buffer composed of 20 mM Tris (American Bioanalytical, Natick, MA), pH 7.4, and 150 mM NaCl (American Bioanalytical). This product, as well as GST and all of the SLC4-related products described later, was collected as single sharp peaks, implying that the products were homogeneous and folded. The CA II protein concentration was determined assuming a molar absorptivity at 280 nm of 5.5 × 10⁴ M⁻¹ cm⁻¹ (18). We assessed CA II purity by denaturing PAGE followed by Coomassie Blue staining, and we verified CO₂ hydration activity using a colorimetric technique (19). Furthermore, we verified the identity of CA II using matrix-assisted laser desorption ionization mass spectrometry (SynPep Corp., Dublin, CA), and 5–7 cycles of Edman N-terminal sequencing (W. M. Keck Proteomics and Mass Spectrometry Facility). Finally, we added glycerol to a concentration of 5% and divided the CA II into aliquots, which we froze on dry ice and stored at −80 °C.

Generation of SLC4-Ct Bacterial Expression Vectors

GST Fusion Proteins—We generated four different cDNA constructs, encoding GST, GST-AE1-Ct, GST-NBCE1-Ct, and GST-NDCBE-Ct, derived from a pGEX-5X-1 bacterial expression vector (GE Healthcare) that encodes GST from the trematode S. japonicum, followed by a factor Xa protease site (IEGR; Fig. 1, A–D) plus 15 residues (Gly… in Fig. 1, A–D) that correspond to the multiple cloning site. Previous groups used this vector to generate GST-AE1-Ct and GST-AE2-Ct for use in binding assays (7–9).

To generate our control GST (GST-IEGRGI in Fig. 1A), we used the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to mutate “CCC” (proline) in the seventh codon following GST to “TAA” (stop codon). To generate GST-AE1-Ct (Fig. 1B), we used PCR to amplify a portion of cDNA (generously provided by Dr. Seth Alper, Beth Israel Hospital, Boston, MA) that encodes CA residues Arg⁸⁷⁷ to Val⁹¹¹ of human erythrocyte AE1 and to insert 5′ and 3′ BamHI restriction sites. We digested the PCR product with BamHI (New England Biolabs, Beverly, MA), purified it with a PCR purification kit (Qiagen, Valencia, CA), and ligated it (T4 DNA ligase; New England Biolabs) the purified product into a linearized (with BamHI) pGEX-5X-1 expression vector. We then used QuikChange to delete the CCC codon (proline) to ensure that GST-AE1-Ct differed from the control GST only by containing residues corresponding to the AE1-Ct.

Using the strategy described above, we generated expression vectors for GST-NBCE1-Ct (Fig. 1C, amplifying Ser⁹⁵¹ to Cys¹⁰³⁵ of human NBCE1-A) and GST-NDCBE-Ct (Fig. 1D, amplifying Ser⁹⁶⁶ to Thr¹⁰⁴⁴ of human NDCBE). In addition, we generated a second version of GST-NDCBE-Ct in which we used QuikChange to add six histidine residues (His₆) immediately after Thr¹⁰⁴⁴, GST-NDCBE-Ct[His₆]. Note that for any of the ligations or QuikChange mutagenesis reactions mentioned above and below, the W. M. Keck DNA Sequencing Center (Yale University School of Medicine, New Haven, CT) sequenced the ligated or mutated region in both the 5′ and 3′ directions.

Pure SLC4-Ct Peptides—We generated four additional cDNA constructs, derived from a pGEX-6X-1 bacterial expression vector (GE Healthcare), which is similar to pGEX-5X-1, except that a PreScission protease (GE Healthcare) cleavage site (PPS) replaced the factor Xa site. The PreScission protease (PP) enzyme is a fusion protein of human rhinovirus type 14 3C protease and GST. We generated cDNA constructs encoding GST-PPS-SLC4-Ct fusion proteins, so that treatment with PP enzyme allowed us to isolate the SLC4-Ct peptide from GST, a strategy used by others to generate pure NBCE1-Ct peptides (20).

To generate cDNA constructs for GST-PPS-AE1-Ct, GST-PPS-NBCE1-Ct, and GST-PPS-NDCBE-Ct, we used an approach similar to that described above for pGEX-5X-1 constructs, except we ligated BamHI-digested PCR products into a pGEX-6X-1 vector linearized with BamHI. Afterward, we deleted codons “CTG” (leucine), “GGA” (glycine), and “TCC” (serine) immediately after the PPS, ensuring that our SLC4-Ct peptides would contain only two non-SLC4-Ct residues (Gly and Pro, required by PP) (Fig. 1, E–G). Additionally, we used QuikChange to generate a His₆ version of each GST-PPS-SLC4-Ct fusion protein, “GST-PPS-SLC4-Ct[His₆].”

Generation and Purification of SLC4-Ct Fusion Proteins and Pure Peptides

We generated the five pGEX-5X-1 and six pGEX-6X-1 fusion proteins using a technique similar to that described for CA II. Both sets of fusion proteins were isolated using GST-Bind resin (Novagen) according to the manufacturer’s protocol.
under reducing conditions (1 mM dithiothreitol (DTT); American Bioanalytical) in BugBuster protein extraction reagent (Novagen) containing protease inhibitors (Complete tablets without EDTA, Roche Applied Science; 1 mM phenylmethylsulfonyl fluoride, American Bioanalytical). From this point, the pGEX-5X-1-based and pGEX-6X-1-based fusion proteins were treated differently, as described below.

*pGEX-5X-1 Fusion Proteins* — To elute GST fusion proteins, we washed the resin with a buffer composed of 50 mM Tris-HCl (American Bioanalytical), pH 8.0, 10 mM reduced glutathione (Calbiochem), and 1 mM DTT. We further purified the GST fusion proteins by gel filtration chromatography as described for CA II, using a Superdex 75 HR 16/60 column (for GST, GST-AE1-Ct, GST-NDCBE-Ct, and GST-NDCBE-Ct[His_{6}]), or a Superdex 200 GL 10/300 column (GE Healthcare; for GST-NBCe1-Ct), equilibrated in a reducing (1 mM DTT) FPLC buffer. We assessed purity by denaturing PAGE followed by Coomassie Blue staining. We estimated the concentrations of the GST fusion proteins from the computed (21) molar absorptivity at 280 nm for GST (4.1 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}), GST-AE1-Ct (4.2 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}), GST-NBCe1-Ct (4.2 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}), GST-NDCBE-Ct (4.9 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}), and GST-NDCBE-Ct[His_{6}] (4.9 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}), and we verified concentrations with a bicinchoninic acid protein assay (Pierce). We stored the material as described for CA II.

**pGEX-6X-1 Pure SLC4-Ct Peptides** — Instead of eluting the GST-PPS-SLC4-Ct fusion proteins, we washed the resin with a buffer composed of 50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA (American Bioanalytical). We then digested the SLC4-Ct from immobilized GST-PPS-SLC4-Ct with PP enzyme (50 units/ml; GE Healthcare) for 16–24 h at 4 °C until no intact GST-PPS-SLC4-Ct was visible by denaturing PAGE with Coomassie Blue staining. After separating the resin from the PP digest (which contained the pure SLC4-Ct peptide), we further purified the pure SLC4-Ct by gel filtration chromatography as described above, using a Superdex 30 16/60 column (GE Healthcare) for AE1-Ct, AE1-Ct[His_{6}], NDCBE-Ct, and NDCBE-Ct[His_{6}] or a Superdex 75 16/60 column for NBCe1-Ct and NBCe1-Ct[His_{6}], equilibrated in a reducing FPLC buffer. We estimated the concentrations of the SLC4-Ct peptides from the computed (21) molar absorptivity at 280 nm for AE1-Ct peptides (1.3 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}), NBCe1-Ct peptides (1.3 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}), and NDCBE-Ct peptides (8.25 \times 10^{3} \text{ M}^{-1} \text{ cm}^{-1}), and we verified the concentrations by amino acid analysis (W. M. Keck Proteomics and Mass Spectrometry Facility, Yale University School of Medicine). We assessed the purity of the peptides with denaturing PAGE followed by Coomassie Blue staining, and we verified the peptide identity by matrix-assisted laser desorption ionization (SynPep Corporation, Dublin, CA) and electrospray (W. M. Keck Proteomics and Mass Spectrometry Facility) mass spectrometry and 5–7 cycles of Edman N-terminal sequencing (W. M. Keck Proteomics and Mass Spectrometry Facility). We stored the material as described for CA II.

**Solid-phase Binding Assays with ELISA Detection**

**Binding of GST-SLC4-Ct to Immobilized CA II** — Our assay was very similar to those previously reported (7–9). We immobilized 200 ng of purified CA II to the bottoms of 48 wells of a 96-well polystyrene High Bind microtiter plate (Corning Glass) in acidic phosphate buffer (APB) composed of 150 mM NaCl and 100 mM Na_{2}HPO_{4} (Fisher), pH 6.0, with gentle rocking for 2 h at room temperature. To the other 48 wells, we added just the APB (without CA II) as a control. After immobilization, we washed the wells three times with a detergent-supplemented phosphate-buffered saline (TPBS) composed of 150 mM NaCl, 5 mM Na_{2}HPO_{4} (Fisher), pH 7.5, and 0.05% Tween 20 (Sigma) using an automated microtiter plate washer (Beckman Coulter, Fullerton, CA), and we then blocked the wells with TPBS containing 2% casein (BioFX Laboratories, Owings Mills, MD) with gentle rocking overnight at 4 °C. The following day, we washed the wells three times with TPBS and added GST and GST-SLC4-Ct (0–1000 nM) in triplicate to separate wells containing immobilized CA II or wells without CA II. The proteins were diluted in a reducing incubation buffer (rIB) containing 100 mM NaCl, 50 mM Tris-HCl at pH 7.5, 5 mM EDTA, 0.25% bovine gelatin (Sigma), 0.05% Triton X-100 (American Bioanalytical), and 1 mM DTT. The plates were incubated with gentle rocking for 20–22 h at room temperature, after which the wells were washed three times with TPBS.

We detected binding of GST-SLC4-Ct to CA II using a colorimetric ELISA, in which we incubated wells for 1 h at room temperature with a polyclonal goat anti-GST antibody (GE Healthcare) diluted 1:20,000 in a nonreducing IB (i.e. rIB without DTT). To at least two wells on each plate, we replaced the anti-GST antibody with a rabbit anti-human CA II polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA) diluted 1:10,000 in IB to verify the presence of CA II. After incubation with primary antibodies, we washed the wells three times with TPBS and incubated with a peroxidase-linked horse anti-goat or goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:10,000 in IB for 1 h at room temperature. After incubation with secondary antibodies, we washed the wells three times with TPBS and developed with a buffer containing 0.05% o-phenylenediamine (Pierce), 22 mM citric acid (Sigma), 54 mM Na_{2}HPO_{4}, and 0.015% H_{2}O_{2} (Fisher) until sufficient color developed. We terminated the color reaction by adding 1 N H_{2}SO_{4} (Fisher) and quantified absorbances at 490 nm using a 96-well plate reader (BMG Labtechnologies, Durham, NC). For each GST-SLC4-Ct fusion protein, the above triplicate experiments were repeated on at least three plates (each plate representing an n of 1).

**Binding of Pure SLC4-Ct Peptides to Immobilized CA II** — Our approach was exactly as described above for the GST-SLC4-Ct assays, except for the following. (i) Instead of adding GST or GST-SLC4-Ct to the wells, we added a pure AE1-Ct (or NBCe1-Ct or NDCBE-Ct[His_{6}]) peptide or its GST-SLC4-Ct counterpart. (ii) We detected binding of the SLC4-Ct and GST-SLC4-Ct to CA II using one of the following primary antibodies: (i) a polyclonal rabbit antibody directed against residues Asp^{906} to Val^{911} of human erythrocyte AE1 (generously provided by Dr. Reinhard Reithmeier, University of Toronto, Canada (22)), diluted 1:6000 in IB; (ii) a polyclonal rabbit antibody directed against the unique Ct (resides Ser^{1034} to Cys^{1072}) of rat NBCe1-A/B “K1A” (23), diluted 1:5000 in IB; and (iii) a mono-
clonal mouse anti-His-Tag antibody (Novagen), diluted 1:1000 in IB.

**Binding of CA II to Immobilized SLC4-Ct Domains**—In a reducing APB (1 mM DTT), we immobilized (in triplicate) 200 ng of GST, GST-SLC4-Ct, or pure SLC4-Ct peptides, or just the APB, to wells of a 96-well microtiter plate. The plates were blocked as described above, incubated with purified CA II (0–1000 nM, diluted in RIB) with gentle rocking for 20–22 h at room temperature, and then washed three times with TBPS. CA II binding to immobilized proteins or pure peptides was detected by ELISA, incubating with the anti-human CA II primary antibody diluted 1:10,000 in IB, washing the wells, and then developing as described above. To three wells on each plate, we replaced the anti-CA II antibody with an anti-GST antibody (for immobilized GST and GST-SLC4-Ct) or an anti-SLC4-Ct antibody (for immobilized pure SLC4-Ct peptides and GST-SLC4-Ct) to verify the presence of the proteins. In some experiments, we detected immobilized AE1-Ct with the “BRIC155” monoclonal antibody (generously provided by Dr. Ashley Toye, University of Bristol, UK), diluted 1:1000 in IB, that recognizes an epitope between residues Phe^{995} and Arg^{1001} of eAE1 (24, 25); we detected immobilized NDBCt-Ct with a polyclonal rabbit antibody “nS11” developed by our group, diluted 1:250 in IB, that is directed against a truncated Ct of human NDBCt (residues Leu^{1001} to Glu^{1027}). For each protein, the above experiments were repeated on three plates.

**Solid-phase Binding Assays with SPR Detection**

All SPR measurements were performed on a Biacore 1000 instrument (Biacore AB, Uppsala, Sweden) in the Biophysics Resource of the Keck Foundation Biotechnology Resource Laboratory (Yale University School of Medicine).

**Binding to Immobilized CA II**—We immobilized CA II (diluted in 10 mM sodium acetate, pH 5.0; Biacore AB) to one of the four flow cell surfaces of a research-grade CM5 sensor chip (Biacore AB) using an amine-coupling kit (Biacore AB), creating a high density surface (~8000 response units (RU)) of CA II. We also created a reference surface by performing the amine-coupling procedure in the absence of CA II on another flow cell surface. We dissolved acetazolamide (ACZ; Sigma) in Dulbecco’s phosphate-buffered saline (Sigma), and sequentially injected this solution over each surface at 100 nM/min for 1 min, followed by a 7-min dissociation interval, similar to previous investigators (26). The pure SLC4-Ct peptides, diluted in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20; Biacore AB) containing 100 μM tris(2-carboxyethyl)phosphine hydrochloride (Sigma), were injected over the CA II and reference surfaces at 25 μl/min for 2.4 min followed by a 5-min dissociation interval.

**Binding to Immobilized SLC4-Ct[His"] Peptides**—To immobilize SLC4-Ct[His"] peptides, we used an indirect capture approach in which we first conjugated the monoclonal mouse anti-His-Tag antibody (diluted in 10 mM sodium acetate, pH 4.0; Biacore AB) to two of the flow cell surfaces of a CM5 sensor chip via amine coupling (~6700 RU). To create an SLC4-Ct peptide surface (~60–100 RU), we injected an SLC4-Ct[His"] peptide (20 μg/ml in HBS-EP) at 25 μl/min for 1.5 min to one of the anti-His surfaces. To create the reference surface, we injected HBS-EP at 25 μl/min for 1.5 min to the other anti-His surface. We diluted the appropriate peptide-specific antibody (i.e. BRIC155, K1A, or nS11) in HBS-EP and injected it sequentially over each surface at 25 μl/min for 2.2 min followed by a 5-min dissociation interval, and we then regenerated the surfaces with two injections of 10 mM glycine (pH 2.0; Biacore AB) at 25–50 μl/min for 1 min. The CA II, diluted in HBS-EP, was then injected over the SLC4-Ct peptide and reference surfaces at 25 μl/min for 2.2 min followed by a 5-min dissociation interval.

**Data Analysis and Statistics**

**Microtiter Plates with Immobilized CA II**—We subtracted the background immunoreactivity in wells without immobilized CA II from the immunoreactivity in the respective wells with immobilized CA II to compensate for nonspecific binding of GST, GST-SLC4-Ct, and pure SLC4-Ct peptides to the plates; this binding was negligible. For plates in which we compared the binding of GST and GST-SLC4-Ct proteins to CA II, we normalized the GST immunoreactivity of each well with immobilized CA II to the mean immunoreactivity of wells incubated with 1000 nM GST. We chose this normalization value because this control condition was represented on all plates, enabling us to compare relative immunoreactivities among plates with different proteins (e.g. GST-AE1-Ct versus GST-NBCe1-Ct). Before determining Kd values and maximal binding (see under “Statistics and Curve Fitting of Microtiter Plate Data”), we subtracted the immunoreactivity of wells incubated with GST from that of wells incubated with GST-SLC4-Ct.

For plates in which we compared the binding of GST-SLC4-Ct and pure SLC4-Ct peptides to CA II, we normalized the absorbance values of all wells with immobilized CA II to the mean absorbance of wells incubated with 1000 nM GST-SLC4-Ct. Because we used a different primary antibody for each SLC4-Ct, we were not able to compare relative immunoreactivities among plates with different SLC4-Ct proteins.

**Microtiter Plates with Immobilized SLC4-Ct Domains**—We subtracted the background immunoreactivity of wells without immobilized GST, GST-SLC4-Ct, or pure SLC4-Ct peptides from the immunoreactivity in the respective wells with immobilized material to compensate for nonspecific binding of CA II to the plates or blocking reagent; this binding was negligible. In addition, we standardized the immunoreactivity of all wells with immobilized material to the average immunoreactivity of wells with immobilized control GST that were incubated with 1000 nM CA II.

**Statistics and Curve Fitting of Microtiter Plate Data**—All data are presented as means ± S.E. An in-house curve-fitting program was used to fit a nonlinear least squares regression to the binding data and estimate the apparent Kd value and maximal binding. To compare mean fitted values among the GST-SLC4-Ct fusion proteins, we used a one-way analysis of variance with a Tukey’s multiple comparison (Graphpad Prism 4; Graphpad Software, San Diego, CA). In some cases, we used a Student’s t test to compare mean immunoreactivities in plates with immobilized SLC4-Ct domains.

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3 L. Virkki, unpublished data.
CA II and SLC4 C-terminal Domains

SPR Data—Transformation, evaluation, and statistical analysis were conducted with BiAevalutai0n software 4.1 (Biacore AB). For a given concentration of analyte (i.e., ACZ, SLC4-Ct peptide, peptide-specific antibody, CA II), ”sensorgrams” for the active surface (i.e., immobilized CA II or SLC4-Ct) and reference surface were x axis-transformed to align the injection points, and y axis-transformed to align the base lines. For each active-surface sensorgram, we then subtracted the respective reference-surface sensorgram to remove nonspecific bulk refractive index changes. We then subtracted the mean response of 2–3 buffer injections from each active-surface sensorgram to correct for drift in the data. To fit kinetic parameters to the ACZ binding data, we used a 1:1 Langmuir binding model that includes a correction for mass transfer effects (26).

RESULTS

We performed solid-phase binding assays using two types of detection, ELISA and SPR. For each, we studied binding in two orientations, with the CA II immobilized and with the SLC4-Ct domain immobilized.

ELISA, Binding of SLC4-Ct Domains to Immobilized CA II

With CA II immobilized, we examined the binding as follows: first of soluble GST fusion proteins using a protocol similar to previous studies (7–9); second of pure peptides. In each case, we examined three SLC4 members as follows: AE1 (SLC4-A1), NBCe1 (SLC4-A4), and NDCBE (SLC4-A8).

GST Fusion Proteins (GST-SLC4-Ct)—We incubated GST and one of three GST-SLC4-Ct fusion proteins (i.e., GST-AE1-Ct, GST-NBCe1-Ct, or GST-NDCBE-Ct) in separate microtiter plate wells, with or without immobilized CA II, and we detected binding using an ELISA for GST. In wells without immobilized CA II, we detected negligible GST immunoreactivity (data not shown), which suggests that the wells and blocking reagent (i.e., casein) do not interact with the GST or any of the three GST-SLC4-Ct constructs. In wells with immobilized CA II, GST binding to immobilized CA II increased with [GST] up to at least 1000 nM (Fig. 2, A–C, open diamonds). However, each of the three GST-SLC4-Ct fusion proteins bound (Fig. 2, A–C, filled symbols) to an even greater extent than GST bound to immobilized CA II. These results qualitatively confirm earlier studies that demonstrated that GST-AE1-Ct, up to concentrations of 200 nM, interacts with immobilized CA II (7–9). In our experiments, the background GST binding to CA II, computed at [GST] and [GST-SLC4-Ct] values of 250 nM, comparable with the highest value used in previous studies (7–9), ranged from ~30% in the NBCe1 experiments to ~60% in the AE1 and NDCBE experiments. Thus, our background values for GST versus GST-AE1-Ct are higher than those reported for GST versus GST-AE1-Ct in previous studies (7–9, 12); we will consider this difference under the “Discussion.”

In Fig. 3, we subtract the background immunoreactivity because of GST (Fig. 2, A–C, open diamonds) from the immunoreactivity for the GST-SLC4-Ct fusion proteins (Fig. 2, A–C, filled symbols) and use a nonlinear least squares approach to fit regression curves to the resulting data. This subtraction reveals saturable binding of the GST-SLC4-Ct proteins to CA II (Fig. 3), as reported by other groups for GST-AE1-Ct (7–9) and GST-NBcn1-Ct (13). We find no significant difference in the apparent affinity ($K_{D}$) for immobilized CA II among the three GST-SLC4-Ct fusion proteins (Table 1). All three of our $K_{D}$ values (322–597 nM) are substantially higher than the “$K_{D}$” value (20 nM) reported previously for GST-AE1-Ct (7). However, as noted under “Discussion,” if we analyze our data using the same approach as others, we indeed obtain $K_{D}$ values in good agreement with theirs.

GST-NBc1-Ct exhibits a significantly higher maximal binding to CA II than either GST-AE1-Ct or GST-NDCE-Ct (Table 1). Possible explanations for this difference are as follows. (i) CA II has more binding sides for GST-NBc1-Ct than for either GST-AE1-Ct or GST-NDCE-Ct. (ii) During the ELISA detection, GST-NBc1-Ct dissociates more slowly from CA II than either GST-AE1-Ct or GST-NDCE-Ct.

Pure Peptides (SLC4-Ct)—These experiments are similar to those above except for two key differences. First, in the micro-
In wells without immobilized CA II, we detected negligible SLC4-Ct (or His-Tag) immunoreactivity, which suggests that the wells and blocking reagent (i.e. casein) do not interact with the SLC4-Ct peptide or GST-SLC4-Ct (data not shown). In wells with immobilized CA II and soluble GST-SLC4-Ct fusion proteins (filled symbols), we detected substantial SLC4-Ct immunoreactivity (Fig. 4, A and B) and His-Tag immunoreactivity (Fig. 4C), the dose dependence of which was very similar to that in corresponding experiments in Fig. 2, A–C (filled symbols). However, we detected no immunoreactivity in wells incubated with the pure SLC4-Ct peptides (Fig. 4, gray symbols). In the cases of AE1 and NBCe1, we detected no binding to immobilized CA II at peptide concentrations as high as 50 μM (data not shown). These results indicate that SLC4-Ct domains bind to immobilized CA II with a relatively high affinity, and dissociate slowly enough to be detected by ELISA, only when presented as a GST fusion protein but not as a pure peptide.

![Graph](image)

**FIGURE 3. ELISA, background-subtracted binding of GST-SLC4-Ct fusion proteins to immobilized CA II.** From the relative GST-SLC4-Ct immunoreactivity data (Fig. 2, A–C, open diamonds), we subtracted the paired values for GST binding from each experiment (Fig. 2, A–C, open diamonds). Values are means ± S.E. The curves through the data represent nonlinear least squares regression fits to the data. GST-NBCe1-Ct (triangles, solid curve) binds immobilized CA II to greater extent than does GST-AE1-Ct (squares, dashed curve) and GST-NDCBE-Ct (circles, dotted curve). For clarity, error bars are omitted when they would overlie the symbol or for concentrations <100 nM.

**TABLE 1**

| Fusion protein | $K_v$ (examination, 0–250 nM) | $K_v$ (examination, 0–1000 nM) | $K_v$ (fitted) | Maximal binding (fitted) |
|---------------|-------------------------------|-------------------------------|----------------|-------------------------|
| GST-AE1-Ct    | 94                            | 264                           | 597 ± 186      | 0.88 ± 0.14             |
| GST-NBCe1-Ct  | 90                            | 191                           | 322 ± 76       | 2.06 ± 0.20*            |
| GST-NDCBE-Ct  | 78                            | 211                           | 330 ± 165      | 0.60 ± 0.13             |

*p < 0.01.

**CA II and SLC4 C-terminal Domains**

In this series of experiments, we reversed the solid-phase binding assay, and we used an ELISA to measure the binding of soluble CA II to immobilized GST, immobilized GST-SLC4-Ct fusion proteins, or immobilized pure SLC4-Ct peptides. In each case, we examined AE1, NBCe1, and NDCBE. This experiment serves two purposes. (i) It represents the physiological orientation of the SLC4-Ct (which is “immobilized” to the inner surface of the cell membrane) and CA II (which is soluble in the cytosol). (ii) It allows us to determine whether the orientation of the proteins influences the dynamics of their interaction.

In wells without immobilized proteins or peptides, we detected negligible CA II immunoreactivity, which suggests that the wells and blocking reagent (i.e. casein) do not interact with CA II (data not shown). In wells with immobilized GST (Fig. 5, A–C, open diamonds) or GST-SLC4-Ct (Fig. 5, A–C, filled symbols), we detected substantial CA II immunoreactivity that increased with increasing amounts of CA II. However, the CA II immunoreactivity in wells with immobilized GST-SLC4-Ct (Fig. 5, A–C, filled symbols) was less than the immunoreactivity of wells with immobilized GST (Fig. 5, A–C, open diamonds). At 1000 nM CA II, binding to immobilized GST was significantly greater ($p < 0.05$) than binding to GST-AE1-Ct and GST-NDCBE-Ct (Fig. 5, A and C) and statistically indistinguishable from GST-NBCe1-Ct (Fig. 5B). In parallel experiments, we verified that equal amounts of GST immunoreactivity were present in wells containing immobilized GST versus GST-SLC4-Ct (data not shown). Thus, the presence of an SLC4-Ct may actually inhibit binding of soluble CA II to immobilized GST, rather than enhance binding, which is opposite to our findings with immobilized CA II (Fig. 2).

In wells with immobilized SLC4-Ct peptides, we detected no CA II immunoreactivity (Fig. 5, A–C, gray symbols). Indeed, for each of the three peptides, we were unable to detect CA II binding even at a CA II concentration of 70 μM (data not shown). Parallel experiments, using anti-SLC4-Ct antibodies, verify that immobiloreactive SLC4-Ct was present and accessible in wells containing GST-SLC4-Ct or SLC4-Ct (data not shown). For both AE1-Ct and NBCe1-Ct, we detected equal SLC4-Ct immunoreactivity for GST fusion protein and the corresponding pure peptide. For NDCBE-Ct, we found ~30% less immunoreactivity for the pure peptide compared with the GST fusion protein. These data suggest that when CA II is soluble it is unable to bind to immobilized Ct domains of SLC4 proteins with a high enough affinity and slow enough dissociation for ELISA detection.

**SPR, Binding of Pure SLC4-Ct Peptides to Immobilized CA II**

In these experiments, we used SPR detection to measure rapid interactions between pure SLC4-Ct peptides and immobilized CA II. As a positive control, we examined the binding of
the CA inhibitor ACZ (222.2 Da). Applying ACZ to immobilized CA II caused a rapid dose-dependent rise in the signal to a maximal response of 110 RU, reflecting the association of ACZ to CA II (Fig. 6A). Conversely, rinsing the CA II surface, after association of ACZ, caused the signal to decay, representing dissociation of the complex. The curves through the data represent the fit of a simple 1:1 Langmuir binding model and predict an equilibrium constant ($K_i$) of 8.7 nM. This value is almost identical to the value of 7 nM reported by Maren (27). Thus, these data verify that the CA II surface is active and that the instrument is capable of detecting the interaction of immobilized CA II with a molecule having a mass as low as 222.2 Da.

Because, other things being equal, the magnitude of the SPR response is proportional to the mass of the analyte, and because the pure SLC4-Ct peptides are 18–45 times more massive than ACZ, their binding to immobilized CA II should produce extremely robust signals. However, the pure SLC4-Ct peptides produced signals that were smaller than those of ACZ and were not dose-dependent (Fig. 6, B–D). The responses are consistent with nonspecific interactions because of bulk refractive index shifts caused by the high concentrations of the peptides. Subsequent denaturing PAGE verifies the presence of intact SLC4 peptides in the analyte solution (data not shown). Thus, our data indicate that the three pure SLC4-Ct peptides do not rapidly associate with immobilized CA II.

**SPR, Binding of CA II to Immobilized SLC4-Ct Peptides**

In these experiments, we used SPR to determine whether CA II rapidly associates with immobilized SLC4-Ct peptides. As a positive control, we examine the binding of peptide-specific
antibodies. Applying an antibody to immobilized SLC4-Ct caused a slow, dose-dependent rise in the signal to a maximal response of ~100 to ~1000 RU (Fig. 7, A–C, insets). Consistent with its poor performance in ELISA assays, the anti-NDCBe1-Ct antibody (αs11) associated weakly and dissociated rapidly. However, in the case of the anti-AE1-Ct antibody (BRIC155) and anti-NBCe1-Ct antibody (K1A), rinsing the SLC4-Ct surface caused at most a slow decay in signal. These data confirm that the SLC4-Ct peptides are immobilized and accessible to relatively large proteins.

Injections of CA II produced signals (Fig. 7, A–C, main panels) that were far smaller than those of the antibodies and that were not dose-dependent. Similar to the SPR data with immobilized CA II in Fig. 6, B–D, the data in Fig. 7, A–C, are consistent with nonspecific signals because of bulk refractive index shifts caused by high concentrations of CA II. Subsequent denaturing PAGE verified the presence of CA II in the analyte solution (data not shown). Thus these data show that CA II does not rapidly associate with immobilized SLC4-Ct peptides.

**DISCUSSION**

In this study, we used two solid-phase binding assays, with ELISA or SPR detection, to address the interaction between CA II and the Ct domains of three SLC4 HCO₃⁻ transporters. The solid-phase binding assay with ELISA detection has been the primary means of analyzing binding of CA II and C termini of SLC4 proteins (e.g. see Refs. 7–9 and 13). However, this assay does not measure real time binding of the proteins because detection (i.e. ELISA) occurs after rinsing off the analyte. On the other hand, the SPR assay detects rapid interactions in real time. Between the two assays, we should have been able to detect an interaction, if it existed.

**ELISA, Interactions between SLC4-Ct Domains and Immobilized CA II**

Background Binding of GST to Immobilized CA II—Previous groups using an assay similar to ours have reported that the background binding of GST to immobilized CA II ranges from <10 to 40% of the binding of GST-SLC4-Ct to CA II (7–9, 12, 28). In this study, this background binding was ~30% of the binding of GST-NBCe1-Ct (Fig. 2B) and ~60% of the binding of GST-AE1-Ct (Fig. 2A) and GST-NDCEBE-Ct (Fig. 2C). Although the range of background GST binding in previous studies is lower than in ours, the "control" GST in previous
studies may have differed from ours. One paper did not specify the identity of the vector encoding GST (28). In four other papers (7–9, 12), the commercial pGEX-5X-1 vector encoding GST was the same as in our study. However, those papers did not specify the location of the stop codon following the GST cDNA. We introduced a stop codon to produce a control GST identical to its GST–SLC4-Ct counterparts, except for the omission of the SLC4-Ct peptide (see Fig. 1, A–D). In the absence of such a stop codon, the Ct of control GST would contain an additional 13 amino acids not present in our control GST or any GST–SLC4-Ct.

**Binding of GST–SLC4-Ct Fusion Proteins to Immobilized CA II**—Although our $K_d$ estimate for GST-AE1-Ct ($\approx 600 \text{ nM}$, Table 1) is noticeably higher than the $K_d$ ($\approx 20 \text{ nM}$) reported previously (7), two major methodological differences could account for our higher estimate. First, the range of GST–AE1-Ct concentrations was 0–1000 nM in our study as opposed to 0–200 nM in the other study. Second, our $K_d$ value is the result of a nonlinear least squares curve fit. In the examination method used to compute $K_d$, one defines “maximum binding” as “the maximum signal obtained in the assay,” which is near the highest examined [GST–AE1-Ct] (i.e. 200 nM). By examining the graph, one then defines $K_d$ as the [GST–AE1-Ct] that produces 50% of the maximum binding. By applying the examination method to our data over a similar concentration range (i.e. 0–250 nM), we obtain a $K_d$ value for GST–AE1-Ct (i.e. 94 nM; see Table 1) that is in better agreement with the other value (7).

Our estimates for the $K_d$ of GST–NBCe1-Ct and GST–NDCBE-Ct (325 nM) to immobilized CA II are somewhat greater than those reported by another group (13) for the binding of GST–NBCn1-Ct to immobilized CA II (101 nM) or an immobilized V143Y mutant of CA II (227 nM); both estimates were obtained over a [GST–NBCn1-Ct] range of 0–200 nM.

**Binding of Pure SLC4-Ct Peptides to Immobilized CA II**—Our data suggest either that SLC4-Ct peptides do not bind directly to immobilized CA II or, if they do, that the dissociation of the pure peptides from CA II is too rapid to allow detection via ELISA.

Previous work (9) showed that a synthetic AE1-Ct peptide prevents binding of 25 nM GST–AE1-Ct to immobilized CA II with a $K_d$ value (i.e. 100 \text{ \mu M}) that is 5000-fold higher than the original $K_d$ value for binding of the membrane domain of AE1 or GST–AE1-Ct to immobilized CA II. Vince and Reithmeier (8) suggested two explanations for the discrepancy. The first is that the pure peptide is not sufficiently well structured to interact stably with CA II but that GST imposes the necessary structure on the Ct of AE1. However, it is not clear how a trematode protein (i.e. GST), coupled via a flexible linker (see Fig. 1B), manages to confer a physiologically relevant structure on human AE1-Ct. Moreover, the PHD program (29, 30) predicts that the secondary structure of pure AE1-Ct is virtually identical to the AE1 component of GST–AE1-Ct.

The second suggested explanation (8) is that the dimerization of GST–AE1-Ct could have allowed the fusion protein to interact with immobilized CA II by a “two-point attachment,” thereby increasing the apparent affinity by a factor of 5000. Presumably, such a two-point attachment would have reduced the dissociation rate by a factor of 5000, allowing detection of binding in their hands even 1 day after rinsing the GST–AE1-Ct from the wells. In the absence of the GST, the off-rate in our experiments would have been 5000-fold higher. Thus, if AE1-Ct and CA II bind to one another but have a high dissociation rate, we may have been unable to detect binding by ELISA, which culminated in our hands 2 h after rinsing the pure AE1-Ct peptides from the wells. However, Vince and Reithmeier (7) demonstrated coimmunoprecipitation of CA II with AE1 from erythrocyte ghosts. These immunoprecipitation experiments involve numerous washes with highly diluted material during the ghosting procedure and are conducted on a long ELISA-like time scale. Therefore, if the direct binding of CA II to AE1-Ct were responsible for the immunoprecipitation results, the complex should have remained intact during the 2-h wash interval of our ELISA. In other words, the requirement for the pure AE1-Ct peptide to dissociate 5000-fold faster than GST–AE1-Ct from immobilized CA II (9) apparently conflicts with the immunoprecipitation data (7).

A way of reconciling the conflict is to propose that the Ct of AE1 may have a different structure when part of intact AE1 than when present as the pure AE1-Ct peptide. Thus, it is impossible to rule out a direct interaction between CA II and the Ct of intact AE1 (or NBCe1 or NDCBE). However, we emphasize that no evidence exists for such a direct interaction. Both Vince et al. (7–9) and the present paper demonstrate a direct interaction between GST–AE1-Ct and immobilized CA II. However, this interaction requires GST, and the secondary structures of the Ct in GST–AE1-Ct the pure AE1-Ct peptide are presumably identical.

**ELISA, Interactions between CA II and Immobilized SLC4-Ct Domains**

Our data indicate that if soluble CA II binds to immobilized SLC4-Ct domains, the dissociation rate is too high for us to detect the interaction using a (slow) ELISA. On the contrary, the only interactions we can detect by a (slow) ELISA suggest that SLC4-Ct domains may actually inhibit binding of CA II to immobilized GST.

Our data are in an apparent conflict with a previous study based on a semi-quantitative pulldown assay with GST–AE1-Ct immobilized to glutathione-Sepharose resin. According to this study, soluble CA II (molecular mass of 29.1 kDa) binds to GST–AE1-Ct (molecular mass of 30.3 kDa), simultaneously detected by denaturing PAGE, to a greater extent than to GST (7), which may have had 13 amino acids not present in the GST–AE1-Ct.

**SPR, Interactions between SLC4-Ct Domains and Immobilized CA II**

To our knowledge, ours was the first attempt to detect an interaction between SLC4-Ct peptides and CA II using SPR technology. Although we easily detected ACZ binding (Fig. 6A), we failed to detect binding of pure SLC4-Ct peptides to the CA II surface (Fig. 6, B and C). These results rule out a rapid association and dissociation between pure SLC4-Ct peptides and immobilized CA II.
Reassessing the Interaction between NBCe1-Ct and CA II

Our ELISA and SPR data for the pure NBCe1-Ct peptide do not corroborate two previous studies. The first used isothermal titration calorimetry to demonstrate that soluble NBCe1-Ct peptides interact with soluble CA II with a relatively high affinity (160 nM) (10). The second used pulldown assays to show binding of soluble NBCe1-Ct peptides to immobilized GST-CA II (11). However, the two previous studies differ from ours in important respects.

Isothermal Titration Calorimetry Study—Compared with the NBCe1-Ct (i.e. Ser951 to Cys1035) in our study, the NBCe1-Ct peptide in the isothermal titration calorimetry study (10) was 36 amino acids longer (i.e. Leu915 to Cys1035) and included part of predicted TM 12, an exofacial loop, and the entire predicted TM 13. The TM 12 moiety contained a third cysteine residue in addition to the two in our NBCe1-Ct construct. Moreover, the authors did not use reducing conditions during the incubation of pure NBCe1-Ct peptides with CA II, raising the possibility that oligomerization via disulfide bridges could have introduced avidity effects, as discussed above for GST-AE1-Ct. We conducted our binding experiments under reducing conditions (i.e. 1 mM DTT or 100 μM tris(2-carboxyethyl)phosphine hydrochloride) to prevent oligomerization.

Pull-down Study—Although the NBCe1-Ct peptide in this study was similar to ours, the experiments were not conducted under reducing conditions (11). Moreover, GST-CA II may have dimerized before attaching to the resin, so that the assay could have measured oligomeric NBCe1-Ct interacting with dimeric CA II complexes. Finally, because this study did not include a control for NBCe1-Ct binding to immobilized GST, the signal could have reflected an interaction of NBCe1-Ct oligomers with GST dimers.

Implications

Interactions between CA II and SLC4-Ct Domains—The preceding review of the literature indicates that all studies reporting an interaction between CA II and an SLC4-Ct involve GST (which is a dimer) and/or nonreducing conditions (which would have allowed the formation of higher order SLC4-Ct oligomers). In addition, our data show that GST and CA II strongly and directly interact as detected by ELISA, regardless of whether one immobilizes CA II or GST, which may explain earlier apparent direct interactions between CA II and the Ct of SLC4 proteins.

A remaining issue is that mutations in the LDADD motif of GST-AE1-Ct, or in the first 17 residues of CA II, eliminate the binding of GST-AE1-Ct to immobilized CA II. The usual assumption is that the binding of pure GST to immobilized CA II is a background phenomenon that does not affect the binding of the Ct of AE1 (which happens to be fused to GST dimers) to CA II. However, many other interpretations are possible. For example, fusing the Ct of AE1 to GST could enhance the direct binding of GST dimers to immobilized CA II. The Ct of AE1 could loop back on the GST dimer and be held in place by an interaction between the GST dimer and the LDADD motif. Thus, LDADD mutations could destabilize the GST-AE1-Ct complex and thereby prevent the Ct of AE1 from enhancing the binding of GST dimers to immobilized CA II.

As to the CA II mutations, the conventional wisdom is that the first 17 residues of CA II interact with AE1-Ct. Here, too, other explanations are possible. For example, the N-terminal of CA II could play a key role in the interaction between CA II and GST dimers. In the final analysis, understanding the interaction between GST-SLC4-Ct and CA II may require a crystal structure.

Interactions between CA II and Other Transporters—Other laboratories have reported binding of CA II to two additional families of acid-base transporters, the SLC26 family of anion exchangers and the SLC9 family of Na+-H+ exchangers. All the binding data comes from solid-phase binding assays with immobilized CA II and ELISA detection.

In the last study, the authors did not use reducing conditions (which would have measured oligomeric NBCe1-Ct interacting with dimeric CA II complexes). Finally, because this study did not include a control for NBCe1-Ct binding to immobilized GST, the signal could have reflected an interaction of NBCe1-Ct oligomers with GST dimers.

CA II and SLC4 C-terminal Domains

Although we detected binding of specific antibodies, our SPR experiments indicate that soluble CA II does not interact with immobilized SLC4-Ct peptides (Fig. 7, A–C). Thus, we are able to rule out the possibility that the interaction between CA II and immobilized SLC4-Ct is rapid and transient.

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Sterling et al. (12) extended the capno metabolon hypothesis by proposing that in an HCO_3^- driven system, CA II enhances AE1 activity. The functional evidence comes from experiments on human embryonic kidney 293 cells heterologously expressing AE1 (12). They found that removing extracellular Cl^- (which causes AE1 to import HCO_3^-) causes a rapid rise in pH_i that they could slow (i) by adding ACZ to block endogenous CA II, (ii) by disrupting the LDADD motif of AE1, or (iii) by heterologously overexpressing an inactive CA II mutant. However, because rates of pH_i change are an indirect measure of HCO_3^- transport, one would expect ACZ to slow the pH_i changes regardless of whether CA II raises AE1 activity per se. Furthermore, the LDADD motif could be so critical to the secondary structure of the intact transporter that mutations to LDADD may inhibit AE1 independent of CA II. Finally, over-expressing mutant CA II might reduce the expression of endogenous CA II. Indeed, Lu et al. (14) demonstrated that CA II, even when directly fused to the NBCe1-Ct, does not enhance the NBCe1 current, which is a direct measure of NBCe1 function. In a theoretical analysis, these authors argued that in an HCO_3^- driven system it was unlikely that the catalytic activity of CA II would substantially enhance the activity of an HCO_3^- or CO_3^-2 transporter.

The Putative CA II-binding Motif—Except for NBCe2 (SLC4A5), the Ct domains of all human SLC4 transporters have a putative CA II-binding motif (e.g. LDADD in AE1), and some transporters (e.g. NBCe1) contain more than one putative motif. Even BTR1, reported to be a borate transporter (39), has a putative CA II-binding motif (LDVMD). Conversely, how is it that NBCe2 can efficiently transport HCO_3^- or CO_3^-2 without a binding site for CA II? The sponge SLC4 homolog has the sequence LDSEE even though it apparently transports silicate (40). Thus, an examination of functional and evolutionary relationships certainly does not support a unique role of LDADD-like motifs for binding α-type carbonic anhydrases like CA II for the purpose of promoting CO_2/HCO_3^- transport.

Interestingly, cytosolic residues that qualify as putative CA II-binding motifs are also found in or near the Ct regions of transmembrane proteins in the aquaporin family (i.e. LDADD in AQP1) and the last intracellular loop of the SLC5 sodium-glucose cotransporter family (e.g. LDAEE in sodium-glucose cotransporters 1 and 2 and IDAEE in sodium-glucose co-transporter 3; see Ref. 41). Therefore, it is possible this motif plays important structural, regulatory, or trafficking roles in certain transmembrane proteins.

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