A Novel Carbonic Anhydrase II Binding Site Regulates NHE1 Activity†

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ABSTRACT: Carbonic anhydrase II (CAII) binds to and regulates transport by the NHE1 isoform of the mammalian Na^+/H^+ exchanger. We localized and characterized the CAII binding region on the C-terminal tail of the Na^+/H^+ exchanger. CAII did not bind to acidic sequences in NHE1 that were similar to the CAII binding site of bicarbonate transporters. Instead, by expressing a variety of fusion proteins of the C-terminal region of the Na^+/H^+ exchanger, we demonstrated that CAII binds to the penultimate group of 13 amino acids of the cytoplasmic tail. Within this region, site-specific mutagenesis demonstrated that amino acids S796 and D797 form part of a novel CAII binding site. Phosphorylation of the C-terminal 26 amino acids by heart cell extracts did not alter CAII binding to this region, but phosphorylation greatly increased CAII binding to a protein containing the C-terminal 182 amino acids of NHE1. This suggested that an upstream region of the cytoplasmic tail acts as an inhibitor of CAII binding to the penultimate group of 13 amino acids. The results demonstrate that a novel phosphorylation-regulated CAII binding site exists in distal amino acids of the NHE1 tail.

The Na^+/H^+ exchanger is a ubiquitously expressed plasma membrane glycoprotein that extrudes one intracellular proton in exchange for one extracellular sodium, thereby protecting cells from intracellular acidification (*1*). The known isoforms of the Na^+/H^+ exchanger have been designated NHE1−NHE9. NHE1[1] was the first isoform cloned (*2*) and is ubiquitously expressed in the plasma membrane of mammalian cells with the other isoforms having more restricted tissue distributions (*3*). In mammals, NHE1 plays a key role in regulation of cell pH, cell volume, and cell proliferation (*1*). It is also critically involved in several forms of heart disease including ischemic and reperfusion damage (*4*) and cardiac hypertrophy (*5*, *6*).

The Na^+/H^+ exchanger (NHE1 isoform) consists of two general domains: a 500 amino acid N-terminal membrane domain and a C-terminal cytoplasmic domain of approximately 300 amino acids. The membrane domain is responsible for ion flux while the cytoplasmic domain regulates activity of the membrane domain (*1*). The large cytoplasmic domain binds to and is regulated by a number of proteins including calcineurin homologous protein (*7*), calmodulin (*8*), and Hsp70 (*9*). In addition the Na^+/H^+ exchanger is subject to regulation by phosphorylation that stimulates transport activity (*10*).

Carbonic anhydrases produce HCO_3^- and H^+ from the hydration of CO_2. Carbonic anhydrase II (CAII) is the predominant cytoplasmic isozyme (*11*). CA activity is required for efficient Cl^-/HCO_3^- exchange by the red blood cell anion exchanger (*12*), and it was demonstrated that CAII binds directly to AE1 and to other bicarbonate dependent transporters (*13*, *14*). The acidic residues (D887ADD) of the C-terminal cytosolic region of the anion exchanger (AE1) bound CAII (*15*).

We recently demonstrated that CAII binds to and modulates the activity of the NHE1 isoform of the Na^+/H^+ exchanger. With exposure of cells to weak acid in the form of CO_2, H^+ transport was enhanced when CAII and NHE1 were coexpressed. CAII bound to the C-terminal 182 amino acids of the Na^+/H^+ exchanger, and phosphorylation enhanced the binding of CAII to NHE1 (*16*). In this report we examine the binding of CAII to the regulatory cytosolic domain of NHE1. We localize the amino acids of NHE1 that bind to CAII and show that mutations that affect CAII binding also affect NHE1 activity. The results support the hypothesis that Na^+/H^+ exchanger activity is linked to bicarbonate based pH regulation through carbonic anhydrase activity.

EXPERIMENTAL PROCEDURES

Materials. GATEWAY cloning items, restriction enzymes, *Escherichia coli* BL21-SI, pDest 14, pDest 15, and pDest 17 were from Life Technologies, Inc. (Rockville, MD). Glutathione-Sepharose 4B was from Pharmacia Biotech AB

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[1] Abbreviations: AE1, anion exchanger 1; BCECF-AM, 2′-7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; CAII, carbonic anhydrase II; C13, C-terminal 13 amino acids of Na^+/H^+ exchanger fused with GST; C26, C-terminal 26 amino acids of Na^+/H^+ exchanger fused with GST; DSP, (dithiobis(succinimidylpropionate)); GST, glutathionine-S-transferase protein; HA, hemagglutinin; His182, polyhistidine fusion of C-terminal 182 amino acids of NHE1; NHE1, Na^+/H^+ exchanger type 1 isoform; P13, penultimate 13 amino acids of Na^+/H^+ exchanger fused with GST; PBS, phosphate buffered saline; pH*i*, intracellular pH; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris buffered saline.

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(Uppsala, Sweden). Glutathione, purified human CAII protein, nigericin, and phenylenediamine were from Sigma (St. Louis, MO). Lipofectamine 2000 was from Invitrogen (Burlington, Ontario). Rabbit anti-human CAII polyclonal antibody was from Abcam Ltd, (Cambridge, U.K.). Conjugated antibodies were purchased from Jackson ImmunoResearch (Mississauga, Ontario), and Ni−NTA agarose resin was from Qiagen (Valencia, CA). DSP (dithiobis(succinimidylpropionate)) was purchased from Pierce, (Rockford, IL). PWO polymerase was from Roche Diagnostics, (Laval, Qc).

Na^+/H^+ Exchanger Fusion Proteins. The carboxyl-terminal 182 amino acids of the human Na^+/H^+ exchanger (NHE1) were expressed as a fusion protein with a C-terminal histidine tag (His182) using the plasmid pDest 14 and the Gateway cloning system as described earlier (*16*). The same cDNA was made again using a mutant form of the Na^+/H^+ exchanger as a template for PCR (*17*). The mutant Na^+/H^+ exchanger had the codons corresponding to ^{753}EEDEDDD^{759} mutated to ^{753}QQNQNNN^{759} resulting in a His182 protein that contained these mutations. The proteins His-156 and His-169 were constructed in a similar manner to His182. The N-terminal primer for PCR was the same (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATGATCCTGAGGAACAACTTGCAGAAGAC-3′); however, the C-terminal primers were designed to terminate at amino acids Q789 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTATTAGTGATGGTGATGGTGATGCTGGGAGCTGGGGCTGTCACTG-3′, 789R) and P802 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCctaCTAttaatggtgatggtgatggtgAGGGTGTGGGCCTGGGTCACTGAGGCAGCGCTGTATCCTCTG-3′, 802R) of the human Na^+/H^+ exchanger, respectively. Amino acids 635−763 of the rabbit NHE1 isoform of the Na^+/H^+ exchanger were made using the same system with rabbit NHE1 cDNA as a template and using the pDest17 expression vector (*18*). The C-terminal 26 amino acids of the human Na^+/H^+ exchanger were expressed and purified as a fusion protein with GST (C26) at the N-terminal (GST-^{790}RIQRCLSDPGPHPEPGEGEPFFPKGQ^{815}). The Gateway cloning system was used as described above, with the pDEST 15 expression vector. The primers used were 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAaGGATACAGCGCTGCCTCAGTGACCCAG-3′ (26F) and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTGCCCCTTGGGGAAGAACGGTTCTCC-3′ (26R). Two variants of this protein were made, one expressing the penultimate group of 13 amino acids (P13) and the other expressing the C-terminal 13 amino acids (C13). To express the penultimate group of 13 amino acids (^{790}RIQRCLSDPGPHP^{802}) the 26F primer was used with the 802R primer in the same system. To express the final 13 amino acids of the Na^+/H^+ exchanger (^{803}EPGEGEPFFPKGQ^{815}) the primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAGCCTGGGGAGGGAGAACCGTTC-3′ (13F) was used with a primer for the very end of the Na^+/H^+ exchanger (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCctaCTAttaatggtgatggtGatggtgCTGCCCCTTGGGGAAGAACGGTTCTCC-3′). All the C-terminal end fusion proteins were expressed in the Gateway system as fusions with GST at the N-terminus as described above.

Mutations were introduced into amino acids 790−803 of the carboxyl terminal of the Na^+/H^+ exchanger. The wild type sequence of ^{790}RIQRCLSDPGPHPE^{803} was mutated to the following ^{790}AIQACLSDPGPHPE^{803} (R790A/R793A), ^{790}RIQRCLANPGPHPE^{803} (S796A/D797N), and 790RIQRCLSDPGPHPQ^{803} (E803Q). Expression and cloning of the mutant proteins was achieved by using overlapping oligonucleotide pairs, filling in with PCR and then cloning the products via the gateway system using the expression vector pDest15 as described above. For some experiments the His182 protein was made as described above; however, site-specific mutagenesis was used to mutate Ser796 to alanine. The mutant protein was produced and purified as described for the wild type His182 protein.

Electrophoresis and Blotting. To examine CAII binding to the Na^+/H^+ exchanger immobilized on nitrocellulose, protein samples were separated on 12% SDS−PAGE and then transferred to nitrocellulose membranes. CAII binding was essentially as described earlier (*16*). Nitrocellulose membranes were blocked with 10% (w/v) skim milk powder in TBS (20 mM Tris, pH 7.4, 137 mM NaCl) overnight at 4 °C. Incubation was with 10 μg of CAII with 1% (w/v) skim milk powder in TBS for 5 h at 4 °C. After washing with TBS (4 × 15 min at room temperature) the nitrocellulose was then incubated with rabbit anti-CAII antibody (1:10 000) in TBS with 1% skim milk powder overnight at 4 °C. A wash with TBS followed for another hour. A subsequent incubation was with goat anti-rabbit-HRP antibodies, and reactive bands were visualized with the Amersham enhanced chemiluminescence system.

To examine protein−protein interactions in a different system, we used a pull down system. GST fusion proteins (20 μg) were incubated with 20 μL of glutathione-Sepharose 4B beads in PBS buffer (140 mM NaCl, 2.7 mM KCl, 12 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 4 °C and rocked overnight. One hundred micrograms of BSA was used to block the beads for 1 h at 4 °C. Then 5 μg of CAII was added into the above solution, and it was rocked for 4 to 5 h at 4 °C. The sample was washed 4 times with PBS buffer (pH 7.4), and then the Na^+/H^+ exchanger -GST-CAII-glutathione-Sepharose complex was pelleted by centrifugation. The pellet was treated with SDS−PAGE loading buffer to elute the proteins from the complex, and after electrophoresis the sample was transferred to the nitrocellulose membranes and blocked with 10% TBS milk. Then the membrane was incubated with anti-CAII antibody (1:10 000) overnight followed by GAR-HRP as a second antibody. Results were typical of at least 3 experiments.

Microtiter Plate Binding Assay. Microtiter plate binding assays were essentially as described earlier (*16*). Purified CAII (0.2 μg/well) was immobilized onto 96 well microtiter plates by overnight incubation in buffer containing 1.25 mg/mL of 1-cyclohexyl-3-(2-morpholinoethy) carbodiimide metho-*p*-toluene sulfonate in 150 mM NaCl, 100 mM sodium phosphate, pH 6.0 at 4 °C. Plates were then washed extensively with PBS (150 mM NaCl, 5 mM sodium phosphate, pH 7.5) and blocked for 1 h at 37 °C in PBS with 0.5% BSA. Plates were washed with PBS and incubated for 1 h with 0−400 nM GST-fusion protein in Ab buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA) at 37 °C. Purified GST was used as a control. Plates were washed with PBS and incubated with rabbit anti-GST (1:4000) antibody for 1 h at 37 °C. Plates were further washed, and goat anti-rabbit horseradish peroxidase (1:4000) antibody was added for 1 h at 37 °C. Red color was

developed by 0.1% *o*-phenylenediamine in substrate buffer (50 mM citric acid, 5 mM sodium phosphate, pH 5.0, 0.09% H_2O_2). The reaction was terminated with 50 μL of 3 M H_2SO_4 per well, and absorbance of microtiter plates was read at A450.

In Vitro Phosphorylation of Proteins. In some experiments cell extracts from rabbit ventricular muscle were used to phosphorylate the His182 fusion proteins. Cell extracts and in vitro phosphorylation of the His tagged and GST tagged fusion protein and C26 were as described earlier (*19*). After phosphorylation (or control mock phosphorylation) by cell extracts, phosphorylated and nonphosphorylated His182 and GST-fusion proteins were used to examine CAII binding to the Na^+/H^+ exchanger immobilized on nitrocellulose as described above. To confirm that equal amounts of phosphorylated and nonphosphorylated protein were present, nitrocellulose transfers were examined by Ponceau S staining. Some in vitro phosphorylation experiments contained [^{32}P] to confirm in vitro phosphorylation of the protein. In these experiments the final ATP concentration was 250 μM. In experiments without [^{32}P] labeling the final ATP concentration was 1 mM. For some experiments the His182 protein or a mutant His182 protein with the Ser796Ala mutation was phosphorylated in vitro with purified protein kinases, either Erk2 ($p42^{mapk}$) or Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) (Biomol). Phosphorylation by Erk2 was using the purified, recombinant, activated kinase (Biomol) in a buffer containing 25 mM HEPES, pH 7.4, 10 mM Mg acetate, 50 μM ATP, 6 μM okadaic acid, 1 mM NaF, and 10 ng/μL Erk in a total volume of 25 μL. In some cases 1 μL of [γ-^{32}P-ATP] was added to the reactions. The reaction was incubated at 30 °C for 90 min with 10 μg of fusion protein. For phosphorylation by CaM kinase II the reaction contained 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 μM ATP, 5 mM $CaCl_2$, 30 μg/mL calmodulin, and 0.8 ng/μL CaM kinase II in a reaction volume of 25 μL. Other incubation conditions were as with Erk, and in both cases reactions were terminated with SDS−PAGE loading buffer prior to electrophoresis and transfer.

Cell Culture and Stable Transfection. AP-1 cells that lack an endogenous Na^+/H^+ exchanger were used to examine Na^+/H^+ exchanger expression and activity. Stable cell lines were made using LIPOFECTAMINE 2000 Reagent according to the manufacturer's protocol as described earlier (*20*). The NHE1 isoform of the Na^+/H^+ exchanger was expressed using an expression plasmid containing a hemagglutinin (HA) tagged human NHE1 isoform of the Na^+/H^+ exchanger. The plasmid pYN4+ (*20*) contains the cDNA of the entire coding region of NHE1. Cells were selected using 800 μg/mL geneticin (G418) and were regularly reestablished from frozen stocks at passage numbers between 5 and 15. For some experiments, cells were cotransfected with the plasmid pJRC36 that encodes human CAII (*16*).

Mutations were made to the plasmid pYN4+ (*20*) using site-directed mutagenesis. Amplification was with PWO DNA polymerase followed by use of the Stratagene (La Jolla, CA) QuikChange site directed mutagenesis kit. Mutations were designed to create a new restriction enzyme site for use in screening transformants. DNA sequencing confirmed the accuracy of the mutations. Amino acids Ser796 and Asp797 were changed to Ala and Asn respectively using the primers S796AD7f 5′-CAGCGCTGCCTCgcTaACCCgGGCCCACACCC-3′ and S796AD7r 5′-GGGTGTGGGCCcGGGTtAgcGAGGCAGCGCTG-3′.

Cell Surface Expression. Cell surface expression was measured as described earlier (*21*). Cells were labeled with Sulpho-NHS-SS-Biotin (Pierce Chemical Company, Rockford, IL), and immobilized streptavidin resin was used to remove cell plasma membrane labeled Na^+/H^+ exchanger. Equivalent amounts of the total and unbound proteins were analyzed by SDS−PAGE and Western blotting against the anti-HA tag as described above. The relative amount of NHE1 on the cell surface was calculated by comparing both the 110 kDa and the 95 kDa species of NHE1 in at least four Western blots of the total and unbound fractions.

Co-immunoprecipitation of NHE1 and CAII. Co-immunoprecipitation of NHE1 and CAII was essentially as described earlier (*16*). All steps were performed at 4 °C unless otherwise noted. Transfected cell lines were washed with phosphate buffered saline (PBS, 150 mM NaCl, 5 mM sodium phosphate, pH 7.4) twice, and then 2 mM DSP was added in 2 mL of cross-link buffer (20 mM Na_2HPO_4, 1.76 mM KH_2PO4, 150 mM NaCl) for 30 min at room temperature. 10 mM Tris was used to quench the reaction for 15 min at room temperature. The mixture was washed with PBS twice and frozen in 2 mL of RIPA buffer in the absence of detergent (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 80 mM NaF, 5 mM EDTA, 1 mM EGTA, 1 mM Na-orthovanadate, and proteinase inhibitor cocktail) by placing cells on dry ice. After defrosting cells, they were sonicated for 15 s. The lysate was centrifuged (35000*g* for 1 h), and the membrane pellet containing NHE1 was resuspended and sonicated for 15 s in 2 mL of RIPA buffer with detergent (1% NP-40, 0.5% deoxychlolate). After further centrifugation (10000*g* for 30 min) the supernatant was collected. The supernatant containing solubilized NHE1 was rocked overnight with 7.5 μL of rabbit anti-HA-tag polyclonal antibody. Protein A Sepharose was added, and the sample was incubated again for 2 h. The resin was washed with RIPA buffer, and protein bound to the resin was solubilized with SDS−PAGE sample buffer. After SDS−PAGE and transfer to nitrocellulose, anti-CAII antibody or anti-HA antibody was used to probe the blots.

Measurement of Intracellular pH. Stably transfected cells were grown on glass coverslips, and the acetoxy methyl ester of 2′-7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) was used to measure intracellular pH essentially as described earlier (*16*). Cells were incubated with BCECF-AM for 18 min at 37 °C, placed into a holder device, and inserted into a fluorescence cuvette at room temperature. The cuvette was initially supplied with 100% O_2 bubbled 5 mM HEPES buffer (pH 7.4 ± 0.05) with a constant flow of 3.5 mL/min and then shifted into a buffer containing 25 mM HCO_3^-. HCO_3^- solutions were bubbled with 5% CO_2/95% air to create dissolved CO_2 (*16*). The intracellular pH was measured using the dual excitation single emission ratio technique with PTI spectrofluorophotometer with excitation wavelengths at 440 and 490 nm and an emission wavelength at 520 nm. The K^+-nigericin technique was used to generate a calibration curve for intracellular dye for each coverslip (*16*). The rate of pH change was calculated using Sigma plot software. For a limited number of experiments where indicated, pH*i* was measured in bicarbonate free medium and cells were acidified with 50 mM ammonium chloride as described earlier (*10*). Where indicated, the activity of the

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634 ILRNNLQKTR QRLRSYNRHT LVADPYEEAW NQMLLRRQKA
#1 [-------------------------------------------
#2 [-------------------------------------------
#3 [-------------------------------------------
#4 [-------------------------------------------
#5 [-------------------------------------------

674 RQLEQKINNY LTVPAHKLDS PTMSRARIGS DPLAYEPKED
 #1 -------------------------------------------
 #2 -------------------------------------------
 #3 -------------------------------------------
 #4 -------------------------------------------
 #5 -------------------------------------------

714 LPVITIDPAS PQSPESVDLV NEELKGKVLG LSRDPAKVAE
 #1 -------------------------------------------
 #2 -------------------------------------------Q
 #3 -------------------------------------------
 #4 -------------------------------------------
 #5 -------------------------------------------

754 EDEDDDGGIM MRSKETSSPG TDDVFTPAPS DSPSSQRIQR
 #1 -------------------------------------------
 #2 QNQNNN--------------------------------------
 #3 -------------------------------------------
 #4 -----------------------------------------]
 #5 ---------]
                                    #6, C26 [----
                                        P13 [----

794 CLSDPGPHPE PGEGEPFFPK GQ 815
 #1 ------------------------]
 #2 ------------------------]
 #3 ---------]
 #6 ------------------------]
P13 ---------]
       C13[---------------]
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FIGURE 1: Amino acid sequence of the C-terminal 182 amino acids of the NHE1 isoform of the human Na^+/H^+ exchanger. Underlined amino acids represent an acidic continuous sequence of amino acids that are similar to the CAII binding site of the Cl^-/HCO_3^- exchangers (*15*). Bold letters represent the penultimate group of 13 amino acids. The amino acids expressed as fusion proteins to localize the CAII binding site are indicated below the sequence. (−) indicates that the amino acid was expressed. #1, His tagged fusion protein of amino acids 634−815 of human NHE1. #2, as in lane 1 except acidic amino acids 753−759 were mutated to neutral amino acids. #3, His tagged fusion protein of amino acids 634−802 of human NHE1. #4, His tagged fusion protein of amino acids 634−789 of human NHE1. #5, His tagged fusion protein of amino acids 635−763 of rabbit NHE1. #6, GST tagged fusion protein of amino acids 790−815 of human NHE1, referred to as C26. P13, GST tagged fusion protein of amino acids 790−802 of human NHE1. C13, GST tagged fusion protein of amino acids 803−815 of human NHE1.

Na^+/H^+ exchanger was corrected for the level of NHE1 expression in different cell lines as described earlier (*21*).

RESULTS

To localize the region of the Na^+/H^+ exchanger that binds CAII we expressed various subfragments of the carboxyl terminal region of NHE1. Figure 1 illustrates the amino acid sequence of the C-terminal 182 amino acids of the human NHE1 isoform of the Na^+/H^+ exchanger. The amino acids expressed in various fusion proteins in this study are indicated. An acidic sequence is underlined. It had similarities to the acidic residues (D887ADD) of the C-terminal cytosolic region of AE1 (*15*) that binds CAII. Figure 2 illustrates the results of experiments in which we examined the ability of CAII protein to bind to various regions of NHE1. Partially

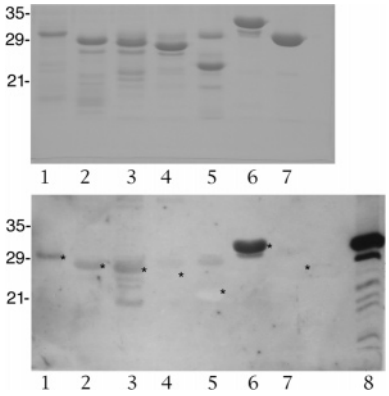

FIGURE 2: Localization of the CAII binding site on the C-terminal region of NHE1. Various subfragments of the NHE1 tail domain were expressed and partially purified as described in Experimental Procedures. They were then transferred to nitrocellulose and used for affinity blotting with CAII as described in Experimental Procedures. Upper panel, coomassie blue stained gel of expressed and purified proteins. Lower panel, affinity blotting with CAII. Lanes 1−6 contained the fusion proteins #1−6 respectively as described in Figure 1. Lane 7, GST protein. Lane 8, purified CAII protein, present as a positive control. In the lower panel the position of the NHE1 fusion proteins is denoted by an asterisk.

purified proteins (upper panel) were overlaid with CAII protein which was detected using anti-CAII protein antibodies as described earlier (*16*). The results of the overlay (lower panel) showed that the fusion proteins of lanes 1−3 and lane 6 bind CAII. Lane 1 contained the C-terminal 182 amino acids of NHE1, and these bound CAII as we have described earlier. We hypothesized that an acidic stretch of amino acids, ^{753}EEDEDDD^{759}, might bind to CAII. However, when we expressed the C-terminal 182 amino acids of NHE1 with this sequence of amino acids mutated to neutral residues, the binding of CAII was not impaired (Figure 2, lane 2). With the mutation of the amino acids there was a slight change in the mobility of the protein, which we have observed earlier (*17*). Lane 3 shows that expression of the C-terminal peptide up to amino acid 802 of human NHE1 produced a protein that could still bind CAII. Other proteins that did not extend past amino acid 789 of the tail did not bind CAII (lanes 4 and 5). A protein of approximately 30 kDa in size was also found to bind CAII (lane 5). The identity of this protein is not known at this time. Expression of the C-terminal 26 amino acids of the Na^+/H^+ exchanger (C26) produced a fusion protein that bound CAII very strongly, while GST alone did not bind CAII. Because expression of the C-terminal 26 amino acids (790−815) resulted in a protein that bound CAII, and because the C-terminal amino acids 634−802 bound CAII, we tentatively assigned CAII binding to amino acids 790−802. Surprisingly the GST fusion of the C-terminal 26 amino acids (C26) bound much more CAII than the same amino acids present in other fusion proteins that contained more proximal regions of the NHE1 tail.

To confirm that the binding of CAII occurred on the penultimate group of 13 amino acids (^{790}RIQRCLSDPGPHP^{802}) of the Na^+/H^+ exchanger tail we used a different procedure. GST-Na^+/H^+ exchanger fusion proteins were produced and incubated with CAII. The resulting complex was then pulled out of solution using glutathione-Sepharose beads. Figure 3A demonstrates the results. When the penultimate group of 13 amino acids of the tail was expressed,

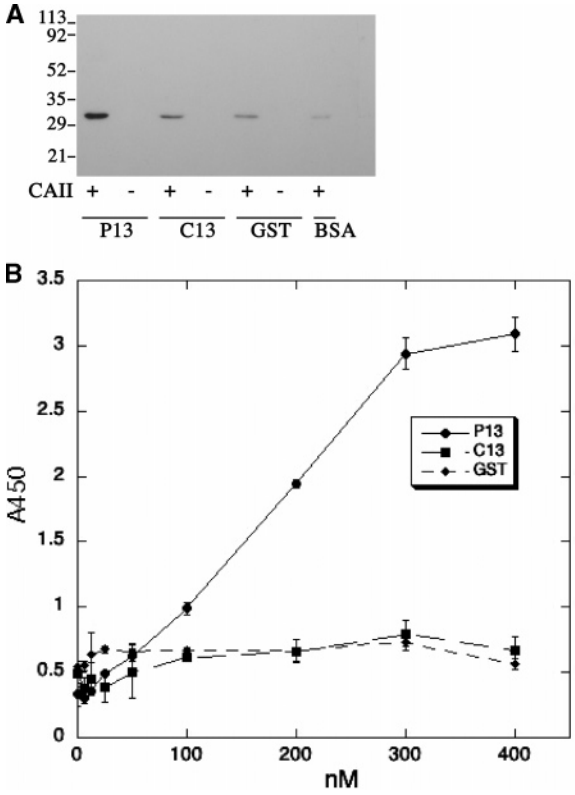

FIGURE 3: Localization of the CAII binding site within the NHE1 C-terminus. (A) Nitrocellulose transfer of Na^+/H^+ exchanger fusion proteins used for pull down assay with CAII. GST fusion proteins were used in a pull down assay with glutathione-Sepharose resin in the presence of CAII. Proteins associated with the resin were separated with SDS−PAGE, transferred to nitrocellulose and probed for the presence of CAII. P13, GST fusion protein expressing the penultimate group of 13 amino acids of the Na^+/H^+ exchanger tail. C13, GST fusion protein expressing the last 13 C-terminal amino acids of the Na^+/H^+ exchanger tail. GST, purified GST protein. Results are typical of three experiments. + or − indicates the presence or absence of CAII added to the pull down assay. BSA was used as a control in the presence of CAII. (B) Solid phase microtiter plate binding assay for Na^+/H^+ exchanger and CAII interactions. CAII was immobilized to microtiter plates as described in Experimental Prodecures. Increasing concentrations of up to 400 nM P13, C13, or GST proteins were added to CAII immobilized to microtiter plates. Bound proteins were detected with anti-GST antibody.

they were able to associate with CAII and the complex was detected in the pull down experiments. Addition of either the C-terminal 13 amino acids (C13), GST, or BSA resulted in only a small background of CAII associated with complex. Nitrocellulose membranes were stained with Ponceau S prior to immunoblotting to confirm that equal amounts of GST or GST-fusion proteins had pelleted with glutathione-Sepharose beads (not shown).

We further confirmed the localization of the CAII Na^+/H^+ exchanger binding region and CAII by using a solid phase binding assay. CAII was immobilized on microtiter plates essentially as described earlier (*16*). The binding curve of P13 to immobilized CAII (Figure 3B) showed that P13 binding increased with increasing concentrations and saturated at higher levels. Under the identical conditions, only a low background binding to GST and C13 was observed. This suggested that the binding was caused by the penultimate 13 amino acids of the Na^+/H^+ exchanger. There was no indication of cooperativity from the shape of the curve.

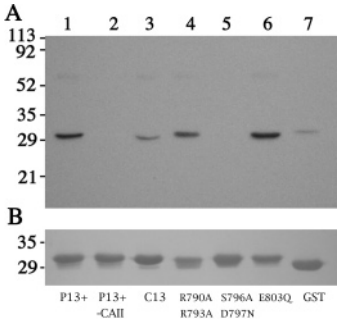

FIGURE 4: Characterization of C-terminal NHE1 amino acids that interact with CAII. (A) Western blot of Na^+/H^+ exchanger fusion proteins used for pull down assay with CAII as described for Figure 3. Samples were probed for the presence of CAII. All samples contained GST or GST fusion protein in the presence of CAII except where indicated. Samples, lane 1, P13+ (GST fusion protein of the sequence ^{790}RIQRCLSDPGPHPE^{803}); lane 2, P13+ pull down in the absence of CAII; lane 3, C13; lane 4, P13+ protein with R790A/R793A mutations; lane 5, P13+ protein with S796A/D797N mutations; lane 6, P13+ protein with E803Q; lane 7, GST. Results are typical of 3 experiments. (B) Ponceau S staining of nitrocellulose transfer indicating the presence of GST or GST fusion proteins.

We next examined which particular amino acids of the P13 region could be important in the binding of CAII to the tail region. GST fusion proteins of the sequence ^{790}RIQRCLSDPGPHPE^{803} were produced. The acidic amino acid E803 was included in these constructs since it was immediately adjacent to the P13 region and acidic amino acids were implicated in the binding of CAII to the anion exchanger (hence the protein is referred to as P13+) (*15*). Figure 4 shows the results of GST pull down experiments. The P13+ protein bound to CAII resulting in an immunoreactive protein that was pulled down with the glutathione Sepharose beads (lane 1). Exclusion of CAII from the reaction resulted in no immunoreactive band (lane 2) and demonstrated that CAII was responsible for the immunoreactivity. The C13 protein bound only trace amounts of CAII (lane 3), equivalent to GST (lane 7). Mutation of amino acids R790 and R793 to alanine did not reduce the binding of CAII (lane 4). However, mutation of amino acids S796 and D797 to alanine and asparagine, respectively, completely eliminated CAII binding (lane 5). Mutation of Glu803 to glutamine did not affect the binding of CAII to this region (lane 6).

We examined the effect of phosphorylation on CAII binding to the C-terminal region of the Na^+/H^+ exchanger. Heart cell extracts were used to phosphorylate a GST fusion protein of the C-terminal 26 amino acids of NHE1, plus GST alone. Figure 5A illustrates an autoradiogram that demonstrates that the cell extracts phosphorylated C26, but did not phosphorylate GST alone appreciably. Figure 5B illustrates GST pull down experiments with phosphorylated and unphosphorylated C26. There was no difference in the amount of CAII associated with C26 in the phosphorylated vs unphosphorylated protein. Figure 5C illustrates Ponceau S staining of the proteins used in Figure 5B, illustrating that an equivalent amount of proteins was used. To compare the effects of phosphorylation in a larger fragment of the NHE1 carboxyl terminal region, we examined the effects of phosphorylation on CAII binding to the His182 protein, that contains the C-terminal 182 amino acids of the Na^+/H^+ exchanger (Figure 5D). Similar to the results we reported previously (*16*), phosphorylation dramatically increased the

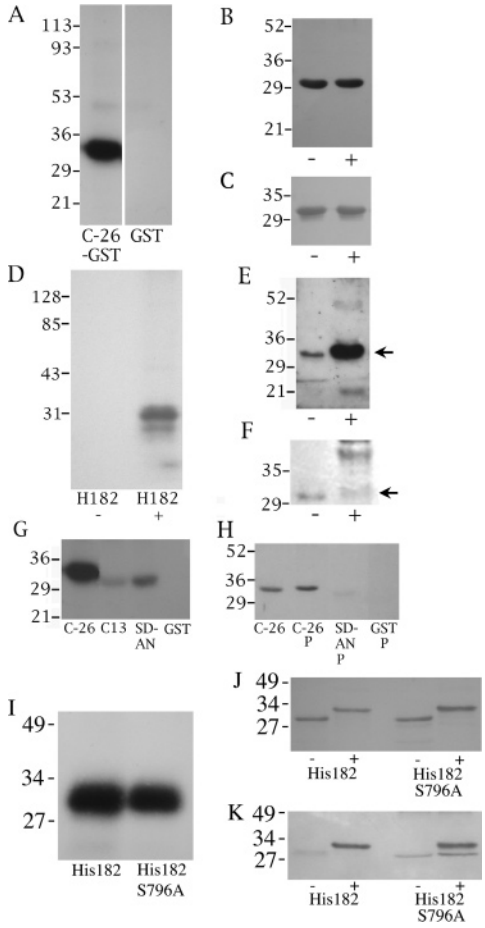

FIGURE 5: Effect of phosphorylation on CAII interaction with the C-terminal 26 amino acids of NHE1. (A) Autoradiogram demonstrating phosphorylation of GST-NHE1 fusion protein. Proteins are the C-terminal 26 amino acids of the Na^+/H^+ exchanger (C26-GST) and GST protein. Cell extracts were used to phosphorylate GST or GST-NHE fusion protein in the presence of [γ-^{32}P]ATP as described in Experimental Procedures. (B) Comparison of levels of CAII binding to phosphorylated and nonphosphorylated C26-GST. Phosphorylated and nonphosphorylated C26-GST were used in a pull down assay with CAII. The figure illustrates samples run on nitrocellulose and probed for the presence of CAII. + indicates C26-GST phosphorylated as described in part A. − indicates unphosphorylated C26-GST protein. (C) Ponceau S staining of C26-GST used for pull down experiments in part B. (D) Autoradiogram demonstrating phosphorylation of His182 (H182) protein. + indicates the presence of cell extracts used in phosphorylation reaction; − indicates their absence. (E) Nitrocellulose transfer of phosphorylated (+) and nonphosphorylated (−) His182 protein used in CAII overlay assay. Arrow indicates the His182 protein. (F) Ponceau S staining of His182 used for CAII overlay assay. + indicates the presence of cell extracts used to phosphorylate His182. Arrow indicates the His182 protein. (G) Autoradiogram illustrating in vitro phosphorylation of Na^+/H^+ exchanger fusion proteins and GST. Cells extracts were used to phosphorylate the proteins C26-GST (C26), C13-GST (C13), P13+ with the S796A/D797N mutation (SD-AN), and GST. (H) Affinity blotting of phosphorylated NHE1 fusion proteins with CAII. Affinity blotting was with CAII as described in Figure 2. Samples were as in Figure 5G with "P" indicating that the proteins were treated under phosphorylating conditions with cell extracts. (I) Autoradiogram demonstrating phosphorylation of His182 protein and His182 protein with S796A mutation. Samples were phosphorylated in vitro in the presence of Erk2 as described in Experimental Procedures. (J) Ponceau S staining of His182 protein and His182 with the S796A mutation, that were used for CAII overlay assay. + indicates that the sample was phosphorylated with Erk2. (K) Affinity blotting of His182 and His182 (S796A) phosphorylated NHE1 fusion proteins with CAII. + indicates that the sample was phosphorylated with Erk2.

level of CAII binding to His182, which was quite small in the unphosphorylated protein (Figure 5E). Figure 5F illustrates the protein used in the experiments. The other proteins present are from the cell extracts used to phosphorylate the His182 protein. In a series of experiments we quantified the level of phosphorylation of the C26 protein, in comparison to the His182 protein. Surprisingly, cell extracts phosphorylated the C26 protein to a higher level (174% ± 23) per mole of protein than His182. The reason for this is not yet clear but could be due to some folding of the His182 protein that restricts accessibility to the kinases.

Figure 5G illustrates the phosphorylation of C26 and several other fusion proteins. C26 was phosphorylated by cell extracts to a relatively high level. C13, consisting of the C-terminal 13 amino acids of NHE1 fused to GST, showed only a very small amount of phosphorylation. Mutant P13+ (S796A/D797N) was barely phosphorylated by cell extracts, and GST showed no phosphorylation by cell extracts. (Wild type P13 was phosphorylated to the same level as C26 (not shown)). Affinity blotting was used to examine the ability of phosphorylation to facilitate CAII binding (Figure 5H). Phosphorylation did not significantly increase CAII binding of C26. Both mutant P13+ (S796A/D797N) and GST treated with phosphorylating conditions did not bind significant amounts of CAII.

We next determined the role of serine 796 in CAII binding in response to phosphorylation by specific protein kinases. We compared the ability of Erk2 ($p42^{mapk}$) and CaM Kinase II to phosphorylate the His182 protein and a mutant His182 protein that had a Ser796Ala mutation. Figure 5I shows that Erk2 phosphorylated both the control His182 protein and the mutant His182,S796A. The level of phosphorylation of the mutant protein was slightly less than that of the control. Figure 5J shows the phosphorylated and nonphosphorylated mutant and control His182 proteins. The phosphorylated proteins showed the characteristic shift in electrophoretic mobility that occurs with phosphorylation. Figure 5K shows CAII binding to both the phosphorylated and unphosphorylated forms of the mutant His182 protein. In both cases CAII bound to the phosphorylated form of the protein and in much smaller amounts to the nonphosphorylated form of the protein. The binding to the phosphorylated form of the mutant His182 with the Ser796Ala mutation was equivalent to the wild type His182 protein. In other parallel experiments we examined the effect of phosphorylation by CaM kinase II. CaM kinase II phosphorylated mutant His182 to only 2/3 the value of the wild type NHE1. In both cases phosphorylation by CaM kinase II did not stimulate CAII binding (not shown).

Since we determined that S796 and D797 are critical to the binding of CAII to the cytosolic tail of the Na^+/H^+ exchanger, we examined the effect of mutation of these residues on the in vivo activity of the protein. Serine 796 and aspartate 797 were mutated to alanine and asparagine, respectively, in the full-length protein. The cDNA encoding the mutant protein was then transfected into the AP-1 cell line in the presence or absence of expressed carbonic anhydrase II. Figure 6A shows a Western blot of stable cell lines made of NHE1 and mutated NHE1 in the presence and absence of CAII. Lanes 1 and 2 are extracts from cell lines expressing the wild type NHE1 protein. Lanes 3 and 4 are extracts from cell lines expressing the SD to AN mutant form

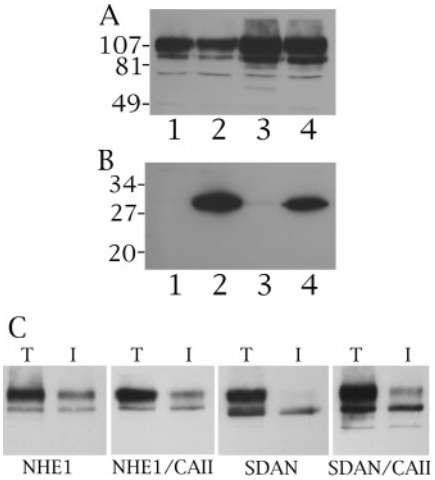

FIGURE 6: Characterization of NHE1 and CAII containing cell lines. (A) Western blot with anti-HA antibody of stable cell lines expressing NHE1 and NHE1 mutant protein in the presence or absence of expressed CAII protein. Lanes 1 and 2, cell extracts from cell lines transfected with wild type NHE1 protein. Lanes 3 and 4, cell extracts from cell lines transfected with mutant (S796A/D797N) NHE1 protein. Lanes 2 and 4 were cotransfected with an expression plasmid for CAII. (B) Western blot of cell lines (as in part A) with anti-CAII antibody. (C) Sulfo-NHE-SS-biotin treated cells were lysed and the solubilized proteins were treated with streptavidin-agarose beads to bind and pull down labeled proteins. Equal amounts of total lysate (T) and unbound lysate (I, intracellular) were run on SDS−PAGE. Western blotting was with anti-HA antibody. NHE1, wild type NHE1 protein; NHE1/CAII, wild type NHE1 coexpressed with CAII; SDAN, mutant (S796A/D797N) NHE1 protein; SDAN/CAII, mutant NHE1 (S796A/D797N) protein coexpressed with CAII.

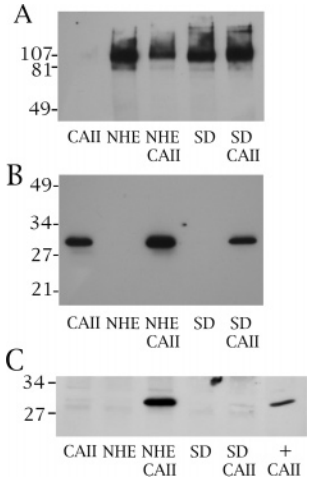

FIGURE 7: Interaction between CAII and wild type NHE1 in vivo. (A) Western blot of immunoprecipitates from cell lines. NHE1 was immunoprecipitated using polyclonal antibody against the HA-tag. The Western blot was probed with monoclonal anti-HA antibody. CAII, cell line transfected with CAII alone; NHE, cell line transfected with NHE1 alone; NHE/CAII, cell line cotransfected with NHE1 and CAII; SD, cell line transfected with mutant (S796A/D797N) NHE1 protein alone; SD/CAII, cell line cotransfected with mutant (S796A/D797N) NHE1 and CAII. (B) Western blot of cell lysates with anti-CAII antibodies. Samples were as in part A. (C) Western blot with anti-CAII antibody of immunoprecipitates made with anti-HA antibody. Samples were as in part A. +CAII indicates exogenous purified CAII added as a positive control for Western blotting.

of NHE1. Panel A demonstrates that all 4 cell lines express the HA-tagged Na^+/H^+ exchanger. Lanes 2 and 4 come from cell extracts that were also transformed with CAII. Panel B demonstrates that these two cell lines both express CAII. We have earlier found that mutation of some amino acids of the Na^+/H^+ exchanger can cause the protein to be targeted to an intracellular location (*20*, *22*). We therefore quantified intracellular localization of NHE1 within AP-1 cells. Cells were treated with sulfo-NHS-SS-biotin, then lysed and solubilized, and labeled proteins were bound to streptavidin-agarose beads. An equal amount of the total cell lysate and unbound lysate was separated by SDS−PAGE followed by Western blotting with anti-HA antibody to identify tagged NHE1 protein. Panel C illustrates examples of analysis of the surface processing of 4 cell lines that expressed either NHE1, NHE1 plus CAII, mutated NHE1 (S796A/D797N), or mutated NHE1 plus CAII. The total lanes (T) and intracellular lanes (I) illustrate the NHE1 protein present. The intracellular lane (I) was that fraction of the Na^+/H^+ exchanger that did not bind to the streptavidin-agarose beads. After quantification of at least four experiments for all cell lines, the percentage of NHE1 localized to the plasma membrane was between 64% and 70% and did not vary significantly between groups.

We next determined if the S796A/D797N mutation of the Na^+/H^+ exchanger affected interactions between CAII and NHE1 in vivo. Cell lines containing either CAII alone, the full length HA-tagged NHE1 protein, CAII and NHE1, the NHE1-S796A/D797N mutant, or the mutant plus CAII were used (*21*). NHE1 was immunoprecipitated from these cell lines using anti-HA antibodies. Figure 7A shows that NHE1 was immunoprecipitated from all the cell lines except the CAII containing cell line that was not transfected with HA-tagged Na^+/H^+ exchanger. Figure 7B is a Western blot of cell lysates of the cell lines, and it demonstrates the presence of CAII in the CAII transfected cells, and in NHE-CAII and mutant NHE1-CAII transfected cell lines. Figure 7C shows the results of immunoblotting of NHE1-HA immunoprecipitates with anti-CAII antibodies. CAII co-immunoprecipitated with wild type Na^+/H^+ exchanger but failed to do so with Na^+/H^+ exchanger with the S796A/D797N mutation. A positive control (+CAII) confirmed the immunoreactivity of the antibodies with CAII.

To examine the effect of the S796A/D797N mutation on the activity of the Na^+/H^+ exchanger in vivo mutant and wild type cell lines were exposed to a weak acid in the form of CO_2 and the rate of recovery was measured as described earlier (*16*). Figure 8A illustrates some examples of the rate of recovery from an acid load in cells expressing wild type Na^+/H^+ exchanger and the S796A/D797N mutant in the presence or absence of CAII. Figure 8B shows a summary of the results corrected for the levels of NHE1 protein expression. Expression of CAII greatly elevated the rate of recovery from an acute acid load. The mutant (S796A/D797N) NHE1 had a reduced rate of recovery while expressing mutant NHE1 (S796A/D797N) and CAII together resulted in activity that was greatly impaired in comparison with the wild type NHE1 and CAII expressed together.

Table 1 summarizes some of the characteristics of the cell lines expressing NHE1 and CAII proteins. The steady state pH*i* of the cells was not significantly different in these cell lines. Similarly the CO_2/HCO_3 induced acid load did not result in varying final pH*i*. The raw rates of recovery of the cells are indicated for the first 60 and 60−120 s time period

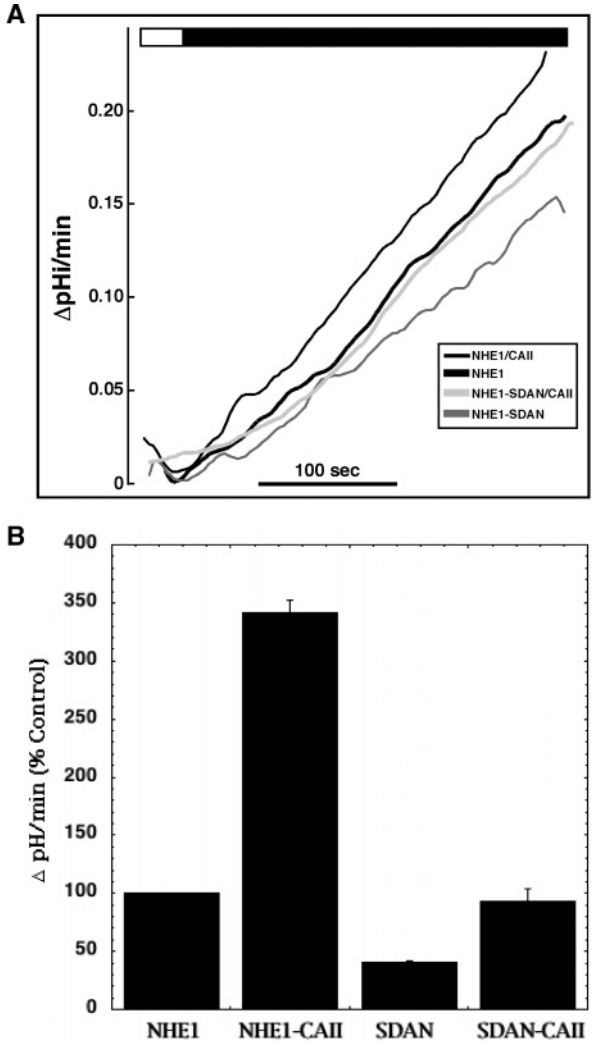

FIGURE 8: Effect of mutation of CAII binding site on Na^+/H^+ exchanger activity in AP1 cells in the presence of bicarbonate. Cell lines were initially bathed in O_2 bubbled HEPES-containing buffer (pH 7.4, open rectangle) and then shifted to CO_2/HCO_3^- containing buffer exposure cells to weak acid in the form of CO_2 (filled rectangle). The recovery from acid load was quantified as described earlier (*16*). (A) Examples of effects on intracellular pH in cell lines expressing mutant or wild type Na^+/H^+ exchanger ± CAII. NHE1/CAII, cell line cotransfected with wild type NHE1 and CAII; NHE1, cell line transfected with wild type NHE1 alone; NHE1-S796A/D797N/CAII, cell line transfected with mutant (S796A/D797N) NHE1 protein and CAII; NHE1-S796A/D797N, cell line transfected with mutant (S796A/D797N) NHE1 and CAII. (B) Summary of H^+ transport rates of NHE and NHE/CAII transfected cells. The activity of the cell lines was corrected for the level of NHE1 protein expression as determined by Western blotting. NHE1, cell line transfected with wild type NHE1 alone; NHE1−CAII, cell line cotransfected with wild type NHE1 and CAII; SDAN, cell line transfected with mutant (S796A/D797N) NHE1 protein; SDAN−CAII, cell line transfected with mutant (S796A/D797N) NHE1 and CAII. Results are the mean ± SE of at least 4 experiments.

after acidification. In both time periods, the rate of recovery was elevated in NHE1/CAII cells while it was not elevated when NHE1 had the SD−AN mutation. In addition coexpression of CAII with mutant NHE1 did not result in elevation of NHE1 activity. In all cases the recovery from acid load was almost completely eliminated by 1 mM amiloride (>90% not shown), indicating that the Na^+/H^+ exchanger was responsible for these activities and not bicarbonate dependent transporters. When an acid load was induced with ammonium chloride in the various cell lines, there was no difference in the rate of recovery from acid load in the absence of bicarbonate (Table 1). The amount of acidification with ammonium chloride was typically to pH 6.0 and did not vary between the cell types.

DISCUSSION

Regulation of the Na^+/H^+ exchanger is a complex process involving protein binding to and phosphorylation of the C-terminal hydrophilic domain. The large cytoplasmic C-terminal domain is known to modulate activity of the membrane domain that transports Na^+ and H^+ ions (*23*). We have previously shown that CAII can bind to and regulate activity of the Na^+/H^+ exchanger. The recovery from exposure of cells to weak acid in the form of CO_2 was stimulated by expression of CAII. In addition, we demonstrated that CAII bound to NHE1 in vivo, that the binding site was present on the C-terminal 182 amino acids of NHE1, and that phosphorylation of the C-terminal increased CAII binding to NHE1 (*16*). Recently, it was also shown that NHE1 and CAII are associated with each other and cause pH microdomains in oligodendrocytes (*24*). In this publication we identified a novel CAII binding region on NHE1 and further characterized the effects of phosphorylation.

CAII has been shown to bind to and regulate activity of the plasma membrane anion exchanger isoform 1 (*14*). The binding site for CAII has been identified as the acidic sequence D887ADD within the 33 residue C-terminal of the anion exchanger protein. At last two negatively charged residues in the charge cluster were required for CAII binding (*15*, *25*). Since we had earlier determined that CAII binding to NHE1 occurs in the C-terminal 182 amino acids, we examined the amino acid sequence of this domain. Amino acids 753−759 (Figure 1) are composed of entirely acidic amino acids. Their similarity to the DADD sequence suggested that this might be the site of CAII binding to the Na^+/H^+ exchanger C-terminal; however, the binding of CAII was not decreased when these amino acids were mutated to neutral residues (Figure 2) and expression of amino acids 634−789, which included the acidic sequence, did not result in a protein that bound CAII. Conversely, expression of the C-terminal 26 amino acids 790−815 resulted in a protein that strongly bound CAII. These results showed that the CAII binding site on the Na^+/H^+ exchanger was not the acidic amino acid sequence from residues 753−759, but was localized to a much more distal region of the protein. We have recently shown that mutation of these acidic amino acids to neutral residues delayed activation of the Na^+/H^+ exchanger protein by ammonium chloride induced acidification, possibly by affecting the conformation of the cytosolic domain. We also showed that this region of the protein can affect calmodulin binding of a more proximal region of the exchanger (*17*). It thus seems that the acidic sequence of the Na^+/H^+ exchanger tail is involved with other aspects of NHE1 function aside from CAII binding.

Since we had localized CA binding to the C-terminal 26 amino acids of NHE1, we further defined the binding site of CAII by expressing and characterizing the C-terminal 13 amino acids and the penultimate 13 amino acids. We found that the C-terminal 13 amino acids did not bind CAII while the penultimate group of 13 amino acids did bind CAII. The

Table 1: Characterization of Stable Cell Lines Expressing NHE1 Mutant NHE1, CAII, and Mutant CAII Proteins[a]

	API/NHE1	API/NHE1/CAII	API/NHE1-SDAN	API/NHE1-SDAN/CAII
steady state pH*i* (HEPES)	7.45 ± 0.06	7.41 ± 0.02	7.47 ± 0.05	7.35 ± 0.03
pH*i* CO_2/HCO_3^- (acid load)	6.97 ± 0.04	7.00 ± 0.03	7.08 ± 0.05	6.99 ± 0.04
ΔpH*i*/min (CO_2/HCO_3^-) (60 s)	0.031 ± 0.005	0.043 ± 0.006[b]	0.024 ± 0.004[b]	0.032 ± 0.006
ΔpH*i*/min (CO_2/HCO_3^-) (60−120 s)	0.031 ± 0.009	0.046 ± 0.005[b]	0.020 ± 0.007[b]	0.035 ± 0.006
ΔpHi/s NH_4Cl (acid load)	.0167 ± 0.023	0.01306 ± 0.004	0.0177 ± 0.0024	0.0158 ± 0.0007

[a] Cell lines were made expressing NHE1, CAII, and mutants as described for Figure 8. CO_2/HCO_3 buffer was used to expose cells to weak acid in the form of CO_2 (Figure 8). Steady state pH was measured for all cell lines as was the initial pH after the acid load. The rate of change of pH over 60 or 120 s was not corrected for the levels of protein expression. For one series of experiments, cells were maintained in bicarbonate free medium and were acidified using ammonium chloride prepulse as described in Experimental Procedures. Values are mean ± SEM, $n = 4$. [b] $P < 0.05$ compared to API/NHE1.

Human	**790**	**RIQRCLSDPGPHP**	**802 (*2*)**
Rabbit	**791**	**RMQRCLSDPGPHP**	**803 (*32*)**
Hamster	**797**	**RIQRCLSDPGPHP**	**809 (*33*)**
Rat	**795**	**RIQRCLSDPGPHP**	**807 (*34*)**
Oncorhynchus	**736**	**RLARCLSDPGPNK**	**748 (*35*)**
Amphiuma	**788**	**RLMRCLSDPGPQP**	**800 (*36*)**
Rabbit NHE3	**713**	**ERELELSDPEEAP**	**725 (*37*)**
Human NHE5	**827**	**VPLHLPSDPRSSF**	**839 (*38*)**

FIGURE 9: Sequence alignment of distal C-terminal amino acids of Na^+/H^+ exchangers. The 13 penultimate amino acids of human NHE are aligned with NHE1 of 5 other species and rabbit NHE3 and human NHE5. Shading indicates amino acids homologous with human NHE1. The amino acids were aligned manually.

C-terminal penultimate group of 13 amino acids are ^{790}RIQRCLSDPGPHP^{802}. The ^{796}SD^{797} to ^{796}AN^{797} mutation eliminated CAII binding in vitro and in vivo and eliminated the ability of CAII to stimulate NHE1 activity in vivo. These results show that the CAII binding site lies within the penultimate 13 amino acids of the Na^+/H^+ exchanger and that the sequence ^{796}SD^{797} forms an essential part of the CAII binding site on the cytoplasmic tail of NHE1.

Mutation of ^{796}SD^{797} to ^{796}AN^{797} of NHE1 eliminated the ability of CAII to enhance NHE1 activity in response to exposure of cells to weak acid in the form of CO_2 (Figure 8). We found that the facilitation of NHE1 activity occurred during the early and later phases of recovery from acidosis. Steady state pH*i* of cells with or without CAII was not altered (Table 1). The physiological role of CAII is thus more related to the recovery from exposure of cells to weak acid in the form of CO_2, rather than steady state pH*i* maintenance. It is of note that, with acidification of cells by ammonium chloride in the absence of HCO_3^-, there was no difference in the rate of recovery between the cells with or without CAII (Table 1). This supports the suggestion that carbonic anhydrase activity was necessary for the facilitation of Na^+/H^+ exchanger activity with exposure of cells to weak acid in the form of CO_2 and that carbonic anhydrase does not simply stimulate NHE1 activity in a nonspecific manner.

Important functional regions of proteins are often conserved across species. Figure 9 examines the conservation of the penultimate group of 13 amino acids of the NHE1 isoform of the Na^+/H^+ exchanger and some other isoforms. In the mammalian NHE1 protein, this region is highly conserved. The core region surrounding amino acids 796 and 797 is also conserved in other vertebrate species such as *Oncorhynchus* and *Amphiuma*. NHE3 had a similar core region in the carboxyl terminal tail while NHE5 had a less conserved core region. Whether these proteins bind to and are regulated by CAII is not known. Other isoforms of Na^+/H^+ exchangers did not possess a similar, conserved region. The sequence "SD" was sometimes found in some isoforms but not in the same carboxyl terminal region and not flanked by leucine and proline residues.

We had earlier found that phosphorylation of the C-terminal 182 amino acids resulted in greatly increased binding of CAII (*16*). However, in this study we found no effect of phosphorylation on CAII binding to a fusion protein containing the C-terminal 26 amino acids (Figure 5B,H). The level of phosphorylation of C26 was greater than that of His182, suggesting that the lack of effect of phosphorylation on CAII binding to C26 was not due to incomplete phosphorylation of C26. We demonstrated that phosphorylation within the C26 region can occur at Ser796 (Figure 5G). However, when we mutated Ser796 individually, without mutation of Asp797, we were still able to get binding of CAII and phosphorylation stimulated binding to the mutant His182 protein (Figure 5I−J). These results indicated that though Ser796 can be phosphorylated in vitro, phosphorylation outside this region is responsible for stimulation of CAII binding.

The suggestion that phosphorylation acts on a proximal site that interacts with C26 is consistent with other results that examined the relative levels of binding of some of the Na^+/H^+ exchanger fusion proteins. A fusion protein consisting of the C-terminal 26 amino acids of NHE1 bound much more CAII than fusion proteins of the entire C-terminal 182 amino acids (Figure 2). These results suggest that another region within the NHE1 C-terminus may reduce CAII binding, possibly by blocking the CAII binding site. Phosphorylation of NHE1 appears to prevent this blockage and causes a large increase in CAII binding. This could be mediated through a change in the conformation of the tail region of the protein or through direct phosphorylation of the region that regulates the CAII binding site.

Ser796 is the only Ser or Thr residue found within C26. Ser796 follows Arg793, and together they make a consensus site for protein kinase mediated phosphorylation by CaM kinase II (*26*). Mutation of both Ser796 and Asp797 eliminated most of the in vitro phosphorylation of C26 by cell extracts. Phosphorylation by CaM kinase II was reduced by mutation of this amino acid; however, more proximal phosphorylation sites outside of C26 appear to be responsible for stimulating CAII binding (Figure 5I−K). It was of interest that Erk mediated phosphorylation could stimulate CAII binding. This is consistent with our earlier results that showed that Erk stimulates NHE1 activity (*19*) and that serum stimulates CAII binding to NHE1 (*16*). However, further experiments are necessary to determine if Erk is the protein kinase regulating CAII binding to NHE1 in vivo.

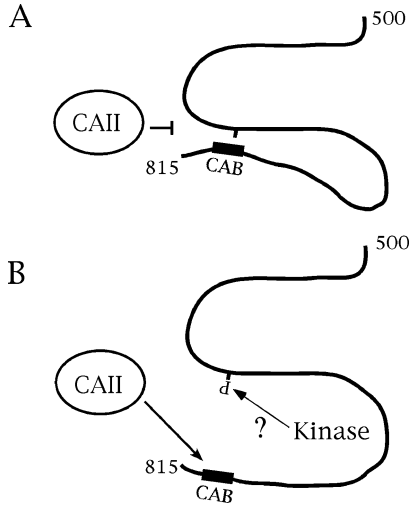

FIGURE 10: Model of phosphorylation regulating CAII binding to the C-terminal of the Na^+/H^+ exchanger. (A) Amino acids 500−815 of NHE1 comprising the cytosolic tail of the protein. The distal CAII binding site (indicated by the solid rectangle) interacts with a more proximal region of the tail, restricting accessibility of the CAII protein. (B) Phosphorylation of the NHE1 cytosolic domain (at site indicated by "P") either affects the structure of the tail causing a distension or directly disrupts an interaction of more proximal amino acids with the penultimate group of 13 amino acids. This allows CAII to bind to the penultimate C-terminal 13 amino acids of the protein. The exact site of kinase mediated phosphorylation is unknown (indicated by "?") but is likely a region proximal to the carbonic anhydrase II binding site. CAB, carbonic anhydrase II binding site.

Figure 10 illustrates a putative model of the regulation of CAII binding by phosphorylation. Supporting the model is our earlier observation that stimulation of intact cells with serum results in a 4−6-fold increase in association of CAII with NHE1 in vivo (*16*). At present, our results suggest that phosphorylation of the more proximal regions of His182 causes the intramolecular regulation of CAII binding. Mutagenesis of Ser796 suggested that this residue was not directly mediating phosphorylation induced effects. It is possible that the site of phosphorylation by protein kinases is the site that directly interacts with the CAII binding site or that phosphorylation causes a change in conformation of the NHE1 tail that makes interactions with the CAII site unfavorable. Future experiments will examine more proximal regions of the NHE1 tail to characterize the intramolecular interactions in more detail.

The family of CA proteins comprises 14 members, and some forms are on the extracytoplasmic face of the membrane (*27*). CAIV exists extracellularly and has been shown to bind to extracellular loop 4 of AE1 (*28*). In addition, CAIV binds to extracellular loop 4 of the sodium bicarbonate cotransporter type I. In this case the binding site was found to contain the sequence RGW and Gly 767 was essential for CAIV binding (*29*). This sequence was not a part of the CAII binding site in this study.

Our findings show that CAII is tethered to the distal region of the NHE1 cytoplasmic tail. In the case of anion exchangers, it has been suggested that a physical interaction with CAII serves to create a more efficient passage of substrate from one enzyme to another in an enzyme chain. This arrangement is referred to as a metabolon (*14*). While this clearly would facilitate transport of bicarbonate out of the cell, it neglects the requisite protons that are produced by the catalytic activity of CAII. The present study shows that CAII is linked to NHE1, in another "metabolon-like" arrangement that is stimulated by phosphorylation. Whether there is a larger complex that involves both the anion exchangers and NHE1 is not yet known at this time. However, it is of interest that some studies have shown linkages in expression and activities between anion exchangers and the Na^+/H^+ exchanger (*30*, *31*). Future studies may explore this in more detail.

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