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A calcineurin homologous protein inhibits GTPase-stimulated Na-H exchange

(Na-H exchanger/Ca^{2+}-binding protein/GTPases)

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ABSTRACT **Activation of the ubiquitously expressed Na-H exchanger, NHE1, results in an increased efflux of intracellular H^+. The increase in intracellular pH associated with this H^+ efflux may contribute to regulating cell proliferation, differentiation, and neoplastic transformation. Although NHE1 activity is stimulated by growth factors and hormones acting through multiple GTPase-mediated pathways, little is known about how the exchanger is directly regulated. Using expression library screening, we identified a novel protein that specifically binds to NHE1 at a site that is critical for growth factor stimulation of exchange activity. This protein is homologous to calcineurin B and calmodulin and is designated CHP for calcineurin B homologous protein. Like NHE1, CHP is widely expressed in human tissues. Transient overexpression of CHP inhibits serum- and GTPase-stimulated NHE1 activity. CHP is a phosphoprotein and expression of constitutively activated GTPases decreases CHP phosphorylation. The phosphorylation state of CHP may therefore be an important signal controlling mitogenic regulation of NHE1.**

Na-H exchangers comprise a family of countertransport proteins catalyzing the electroneutral exchange of Na^+ and H^+. The only ubiquitously expressed isoform, NHE1, functions primarily in pH_i homeostasis and cell volume regulation. Increases in NHE1 can contribute to increases in pH_i and provide either a permissive or an obligatory signal for cell proliferation (1, 2) and differentiation (3). Increased NHE1 activity is also associated with pathological conditions, such as neoplastic transformation (4–6), tumor invasion (7), and essential hypertension (8, 9).

A distinct characteristic of NHE1 is that multiple extracellular signals modulate its activity. Osmotic stress (10, 11) and cytokines (12) stimulate the exchanger, as do growth factors acting at receptor tyrosine kinases (13) and hormones and neurotransmitters acting at seven transmembrane receptors (13, 14). Low molecular weight GTPases of the Ras (7, 15) and Rho (15) family, as well as α subunits of heterotrimeric GTPases (16–18), stimulate NHE1 activity through multiple intracellular signaling pathways (15, 17, 19). Ultimately, these distinct pathways must converge on the exchanger or on an upstream regulator of the exchanger. Little is known, however, about proteins that directly interact with NHE1 and regulate its activity.

The secondary structure of NHE1 contains 10–12 transmembrane domains and a large (300-aa) C-terminal cytoplasmic regulatory domain (19, 20). The transmembrane domains are the site of functional ion exchange and amiloride sensitivity and contain a H^+ modifier site conferring pH sensitivity (21). The cytoplasmic regulatory domain determines the set point value of the exchanger and confers regulation by growth factors (13, 21), hormones (13, 22), and osmotic stress (11, 12). Although multiple signaling pathways regulate the exchanger, calmodulin is the only protein that has been reported to bind to this regulatory domain and modulate NHE1 activity (23, 24). We therefore sought to determine whether additional proteins interact directly with the exchanger. Using a glutathione *S*-transferase (GST) fusion protein of the NHE1 C-terminal cytoplasmic domain (amino acids 503–815) as an hybridization probe to screen a λgt11 expression library, we identified a protein, CHP (for calcineurin B homologous protein), that binds to NHE1 and regulates its activity. CHP specifically associates with NHE1 *in vitro* and *in vivo* and regulates GTPase activation of the exchanger.

MATERIALS AND METHODS

DNA Constructs, Cell Culture, and Transfection. Mutationally active forms of Ras-V12, Gα13 (α13Q226L), $G\alpha_s$ (α_sQ227L), RhoA (RhoAV14), and Cdc42 (Cdc42V14) were constructed as described (15, 16, 25). Transient expression of these GTPases was obtained using the Lipofectamine reagent (GIBCO/BRL). COS-7 and CCL39 cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% or 5% fetal bovine serum, respectively.

Construction and Purification of GST–NHE1 Fusion Proteins. cDNAs encoding the human C-terminal cytoplasmic domain (amino acids 503–815) of NHE1 and the indicated subdomains of NHE1 were cloned into a pGEX-2TK vector for the production of GST fusion proteins from bacteria (Pharmacia). GST fusion proteins were produced and purified by glutathione-Sepharose 4B beads (Pharmacia) according to the manufacturer's instructions.

Screening of λgt 11 Expression Library and DNA Sequence Analysis. Purified GST and GST–NHE1 fusion proteins were [γ-^{32}P]ATP-labeled with protein kinase A (Sigma), which specifically recognizes the human heart muscle kinase (HMK) phosphorylation site in the pGEX-2TK vector (26, 27). A human B-cell cDNA library constructed in λgt 11 was screened and a positive clone, interacting with GST–NHE1 but not GST alone, was purified by sequential hybridizations (26, 27). A cDNA insert of the positive clone was recovered and sequenced using the United States Biochemical Sequenase sequencing kit. A homology search for the CHP sequence was performed in the protein data base, and sequences were aligned by the GCG program.

Northern Blot Analysis. Total RNA (10 μg) from human fetal tissues (15th week of gestation) were electrophoresed in a 1% agarose gel, transferred to Nytran membrane (Schleicher & Schuell) and hybridized with a [γ-^{32}P]ATP-labeled, *Bgl*II-

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Abbreviations: GST, glutathione *S*-transferase; CaM, calmodulin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U61538).

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digested CHP cDNA fragment under high-stringency conditions (28).

***In Vitro* $^{45}Ca^{2+}$-Binding Assay.** CHP was expressed and purified from bacteria as a GST fusion protein (Pharmacia). The binding of $^{45}Ca^{2+}$ to GST–CHP fusion proteins was assayed as described (29). Briefly, purified GST and GST–CHP fusion proteins were resolved by SDS/PAGE and transferred to nitrocellulose. The membrane was washed two times for 10 min in 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl (pH 6.8) before incubation for 10 min with 1 mCi (1 Ci = 37 GBq) of $^{45}CaCl_2$ per ml in the same buffer. The binding of $^{45}Ca^{2+}$ was visualized by autoradiography.

***In Vitro* CHP and NHE1 Binding Assay.** CHP was *in vitro*-transcribed/translated and ^{35}S-labeled using the TNT-coupled rabbit reticulocyte lysate system according to the protocol of the manufacturer (Promega). GST–NHE1 fusion proteins were purified from bacteria and adsorbed to glutathione-Sepharose 4B beads. Binding of [^{35}S]CHP to GST–NHE1 constructs adsorbed on Sepharose 4B beads was performed in 50 mM Tris·HCl (pH 7.5), 120 mM NaCl, 2 mM EDTA, and 0.1% Nonidet P-40 for 1 h at 4°C. After several washes, the bound complex, as well as the *in vitro*-translated CHP product (positive control), were examined by SDS/PAGE and visualized by autoradiography.

Expression and Coimmunoprecipitation of CHP and NHE1 *in Vivo*. CHP and NHE1 were Myc- and Glu-Glu (EE)- tagged at their C-termini, respectively, subcloned into pcDNA1 (Invitrogen), and transiently expressed alone or coexpressed in COS-7 cells. Coimmunoprecipitation of CHP and NHE1 was performed as described (30). Briefly, total cell lysates from the [^{35}S]methionine-labeled cells were used for the first immunoprecipitation of CHP with anti-Myc antibodies, and the immunoprecipitation products were disrupted by boiling and subjected to a second immunoprecipitation with anti-EE antibodies. Immunoprecipitated products were electrophoresed on SDS/PAGE and visualized by autoradiography.

***In Vivo* CHP Phosphorylation Assay.** CCL39 cells transiently expressing Myc-tagged CHP and mutationally activated GTPases were labeled for 16 h in 3 ml of serum-free DMEM containing 100 μCi of [^{32}P]orthophosphate per ml. Serum (10%) was added 15 min before lysis. The cells were lysed in a buffer containing 20 mM Tris·HCl (pH 7.6), 130 mM NaCl, 3 mM KCl, 25 mM sodium pyrophosphate, 5 mM EDTA, 2 mM ATP, and 1% Nonidet P-40. Total cell lysates were used for the immunoprecipitation of CHP with anti-Myc antibodies. The immunoprecipitated CHP was electrophoresed on SDS/PAGE and visualized by autoradiography.

Measurement of NHE1 Activity. NHE1 activity was determined in populations of CCL39 cells. Cells were plated on glass coverslips 14–18 h after transfection, allowed to adhere, and maintained in the absence of serum for an additional 18 h. NHE1 activity was determined in a nominally HCO_3^--free Hepes-buffered medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 1 mM KH_2PO_4, 10 mM glucose, and 30 mM Hepes, titrated to a pH of 7.4. Cells were loaded with 1 μM acetoxy-methyl ester derivative of the pH-sensitive dye 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF; Molecular Probes) for 15 min at 37°C without CO_2. Coverslips were transferred to polystyrene cuvettes modified to allow a continuous flow-through at a rate of 2 ml/min. Cuvettes were placed in a thermostatically controlled (37°C) holder in a Shimadzu RF5000 spectrofluorometer. BCECF fluorescence was measured by alternately exciting the dye at 500 and 440 nm at a constant emission of 530 nm (31). Fluorescence ratios were calibrated with 10 μM nigericin in 105 mM KCl (32), and pH_i was determined using a conversion program developed by G. Boyarsky (University of Texas, Galveston). Cells were acid-loaded by the application (10 min) and removal of 20 mM NH_4Cl (20 mM NaCl replaced with 20 mM NH_4^+/NH_3) (33). Rates of recovery from this acid load [dpH_i/dt] were determined by evaluating the derivative of the slope of the pH_i tracing at pH_i intervals of 0.05.

RESULTS AND DISCUSSION

To identify NHE1-interacting proteins, a GST fusion protein of the NHE1 C-terminal cytoplasmic domain (amino acids 503–815) was used as an hybridization probe to screen a human B-cell cDNA library constructed in a λgt11 expression vector (26, 27). One clone that specifically interacted with the GST–NHE1 fusion protein, but not with GST alone, was identified. The cDNA insert was recovered and sequenced. This clone contained a partial cDNA that was used to isolate a cDNA clone with a complete open reading frame from a second human B-cell library. Sequence analysis revealed that this positive clone encodes a protein of 195 aa with two distinct and two potential EF-hand motifs (Fig. 1*A*, underlined). The structural features of the helix-loop-helix of the EF-hand, as well as the calcium-chelating residues, conform to the consensus sequences for functional Ca^{2+}-binding motifs, as reviewed by Strynadka and James (34). The identified protein shares the highest homology with calcineurin B, the Ca^{2+}-binding subunit of the heterotrimeric phosphatase calcineurin (65% similarity and 43% identity), and with calmodulin (CaM; 59% similarity and 33% identity; Fig. 1*B*), suggesting it is a Ca^{2+}-binding protein. We therefore named this protein CHP, or calcineurin B homologous protein. The Ca^{2+}-binding capacity of CHP was confirmed by an *in vitro* $^{45}Ca^{2+}$-binding assay with purified GST–CHP fusion proteins (Fig. 1*C*). GST–CHP fusion proteins, but not GST alone, bound $^{45}Ca^{2+}$.

During the preparation of this manuscript, Barroso *et al.* (35) identified a novel Ca^{2+}-binding protein, p22, obtained from a rat liver cDNA library. p22 shares 99% amino acid sequence identity with CHP; therefore, we speculate p22 and CHP are Ca^{2+}-binding proteins of the same origin but expressed in different species. p22 mRNA was widely expressed in rat tissues as a 2.2- to 2.4-kb transcript (35). Northern blot analysis demonstrates that CHP is also widely expressed in human tissues, but as a single 4-kb transcript (Fig. 1*D*). This distribution of CHP correlates with the ubiquitous expression of NHE1.

In vitro-translated CHP specifically interacted with a GST fusion of the complete C-terminal domain of NHE1 (amino acids 503–815), but not with GST alone (Fig. 2*B*), indicating NHE1 and CHP associate directly. Previous studies have indicated that within this C-terminal domain, there are at least three distinct subdomains that function in regulating exchange activity (ref. 13; Fig. 2*A*). The distal C-terminal subdomain (amino acids 691–815) contains a number of serine residues that are phosphorylated upon growth factor activation of the exchanger (36). Deletion of this subdomain by a C-terminal truncation at amino acid residue 691 (37) or functional blockade of this domain with site-specific antibodies (22) only partially inhibits activation of the exchanger. Hence, although changes in the set point of the exchanger may be partially mediated by phosphorylation of this distal subdomain, additional phosphorylation-independent regulatory sites are also important. A second regulatory subdomain (amino acids 637–691) binds calmodulin and may be important in NHE1 regulation by Ca^{2+}-mediated signaling mechanisms (23, 24). The third functional subdomain (amino acids 567–637) encompasses residues that are critical for growth factor-stimulated NHE1 activity. Deletion of this subdomain completely abolishes growth factor activation (37). To localize the specific CHP-binding domain on NHE1, *in vitro*-translated CHP was incubated with GST fusions of a set of NHE1 deletion constructs. CHP did not bind to the extreme C terminus subdomain (Fig. 2*B*, amino acids 672–815) or to the CaM-regulated subdomain (Fig. 2*B*, amino acids 637–691), indicating that CHP and CaM bind to different regions of NHE1.

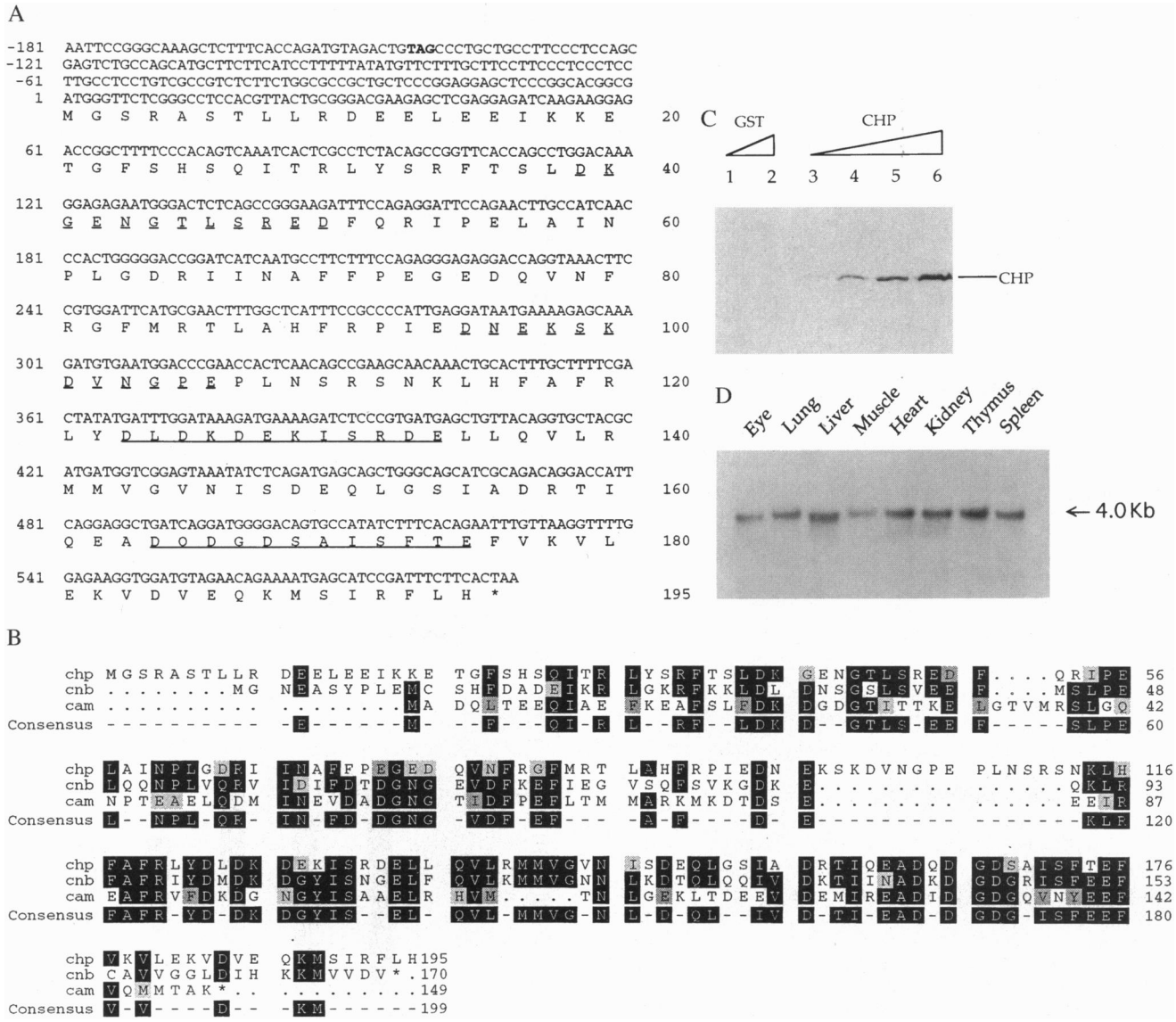

FIG. 1. Molecular characterization of CHP. (*A*) Nucleotide and deduced amino acid sequences of CHP. The two distinct and two potential EF-hand Ca^{2+}-binding motifs are underlined with a solid line and a dashed line, respectively. The stop codon is marked with an asterisk. (*B*) Amino acid sequence alignment between CHP, calcineurin B (cnb), and CaM (cam). Dots represent gaps for optimal alignment. Identical amino acids are in black boxes; conservative substitutions are in gray boxes. The sequence labeled consensus shows the residues present in at least two of the three sequences. (*C*) $^{45}Ca^{2+}$ binding of CHP *in vitro*. Purified GST–CHP fusion protein (0.5 μg, 1 μg, 2 μg, and 5 μg in lanes 3, 4, 5, and 6, respectively) binds $^{45}Ca^{2+}$; 5 μg and 10 μg of GST control does not bind $^{45}Ca^{2+}$ (lanes 1 and 2, respectively). (*D*) CHP gene expression in human fetal tissues. A single transcript of 4 kb (arrow) is detected in eye, lung, liver, muscle, heart, kidney, thymus, and spleen (lanes 1, 2, 3, 4, 5, 6, 7, and 8, respectively).

CHP did, however, bind to the third regulatory subdomain (Fig. 2*B*, amino acids 567–637), which is critical for growth factor regulation of NHE1 (37). Although an accessory protein has been postulated to interact with this site (13), the identity of this putative NHE1 regulatory protein has not been determined. Whether CHP is the only NHE1-interacting protein that binds to this critical regulatory subdomain remains to be determined.

The specific association between CHP and full-length NHE1 *in vivo* was confirmed by coimmunoprecipitating Myc-tagged CHP and Glu-Glu (EE)-tagged NHE1 expressed in COS-7 cells. Lysates from ^{35}S-metabolically labeled cells were subjected to a first immunoprecipitation with anti-Myc antibodies (Fig. 2*C*, lanes 1–3). The precipitated complexes were then disrupted and subjected to a second immunoprecipitation with anti-EE antibodies (Fig. 2*C*, lanes 4–6). NHE1 coimmunoprecipitated with CHP (Fig. 2*C*, lanes 3 and 6) and vice versa (data not shown).

To determine the functional importance of a CHP–NHE1 association, we transiently overexpressed wild-type CHP in CCL39 fibroblasts and measured NHE1 activity by determining the rate of pH_i recovery from an NH_4Cl-induced acid load (31). In a Hepes buffer, the acute pH_i recovery from an acid load in CCL39 cells is due exclusively to acid extrusion from NHE1 (15). In quiescent cells, maintained in serum-free medium for 18 h, overexpression of CHP had no effect on resting pH_i (7.01 ± 0.02 in vector controls and 7.00 ± 0.02 in the presence of CHP) or on the rate of pH_i recovery from an acid load [16.63 ± 1.01 $dpH_i/dt \times 10^{-4}$ pH/s at pH_i 6.75 in vector controls and 16.16 ± 1.04 in the presence of CHP; n = seven separate transfections]. In contrast, overexpression of CHP inhibited serum-stimulated pH_i recovery (Fig. 3*A*) and reduced serum-induced increases in resting pH_i from 7.19 ± 0.02 in vector controls to 7.02 ± 0.03 in the presence of CHP.

Overexpression of CHP also inhibited NHE1 activation by GTPase-deficient RasV12 and Gα13Q226L (Fig. 3*B*). Coex-

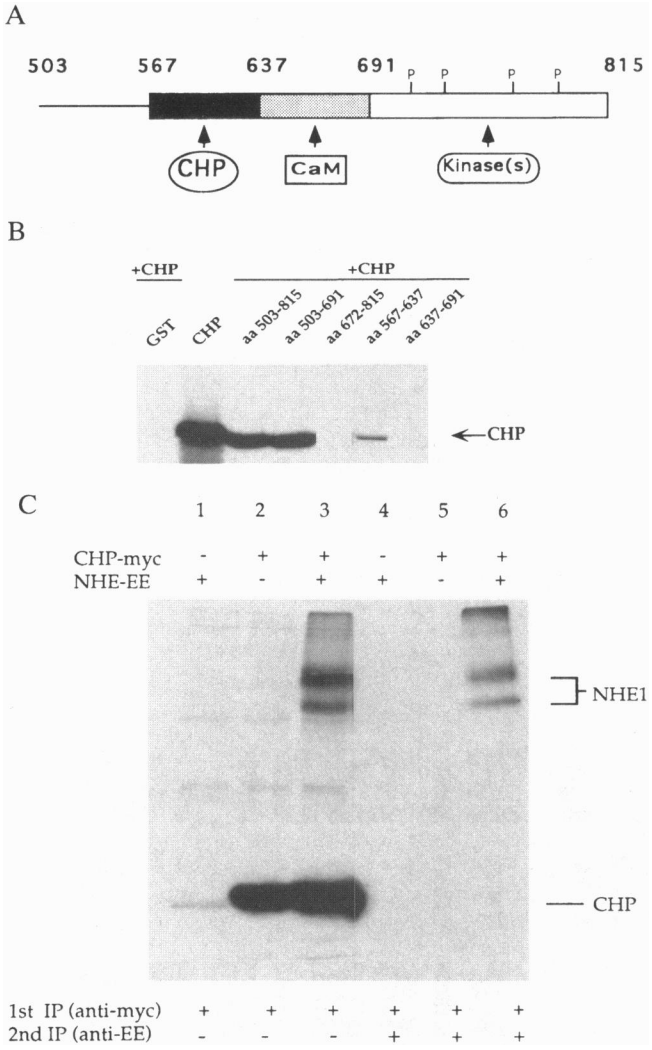

FIG. 2. *In vitro* and *in vivo* association of CHP with NHE1. (*A*) Functional domains and CHP binding site within the C-terminal cytoplasmic domain of NHE1. (*B*) *In vitro* binding of CHP to the cytoplasmic domain of NHE1 and a set of NHE1 deletion constructs. *In vitro*-translated, [^{35}S]CHP was incubated with GST, GST–NHE1 (amino acids 503–815), and NHE1 constructs, as follows: NHE1 amino acids 503–672; NHE1 amino acids 672–815; NHE1 amino acids 567–637; and NHE1 amino acids 637–691. The adsorbed proteins were separated by SDS/PAGE. (*C*) Association of CHP with full-length NHE1 *in vivo*. COS-7 cells, plated on 100-mm dishes, were transfected with 5 μg of expression plasmids of EE-tagged NHE1 alone (lanes 1 and 4), Myc-tagged CHP alone (lanes 2 and 5), or both (lanes 3 and 6). Lysates from ^{35}S-labeled cells were subjected to a first immunoprecipitation with anti-Myc antibodies, and the immunoprecipitation products were disrupted by boiling and subjected to a second immunoprecipitation with anti-EE antibodies (lanes 4–6). NHE1 coprecipitated with CHP (lanes 3 and 6, marked bands). The two NHE1 bands most likely represent the glycosylated and unglycosylated forms of NHE1.

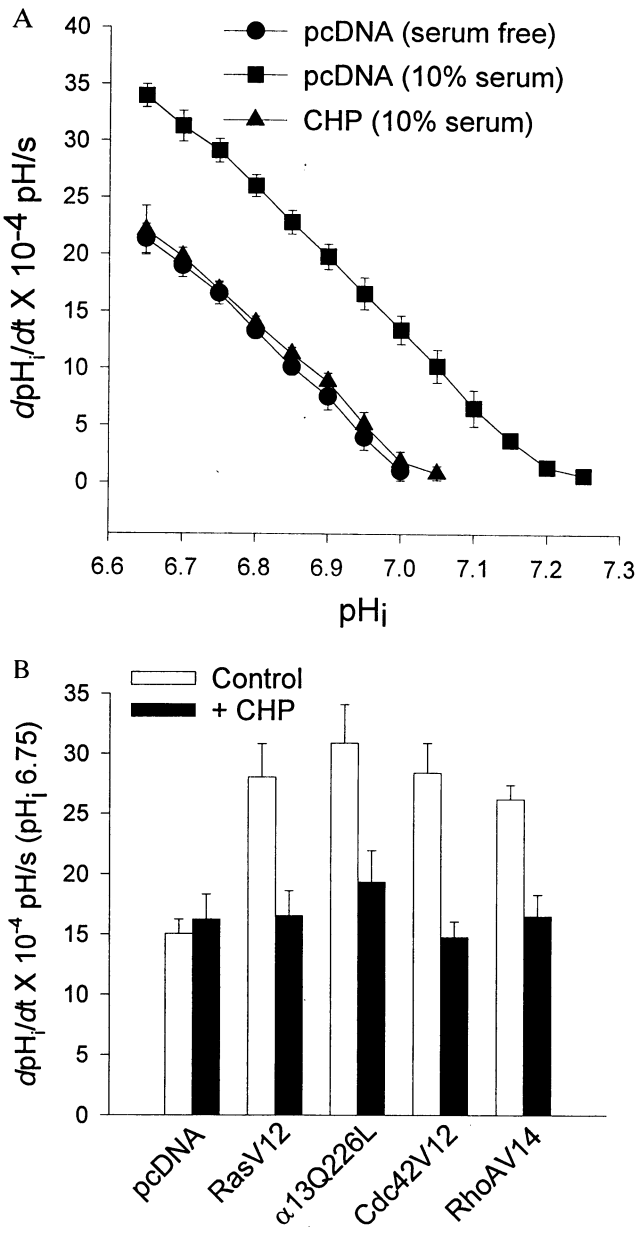

FIG. 3. Effects of CHP on NHE1 activity. (*A*) CHP inhibited serum-stimulated acid extrusion. The rate of pH_i recovery [$d(pH_i)/dt \times 10^{-4}$ pH/s] from an NH_4Cl-induced acid load was determined in CCL39 cells transiently expressing empty vector (pcDNA) or CHP in the absence or presence of serum (10% for 15 min). Data represent the means ± SEM of recovery rates in five separate transfections. (*B*) CHP-inhibited acid extrusion stimulated by mutationally activated GTPases. Data represent the means ± SEM of recovery rates at pH_i 6.75 in three independent transfections with each GTPase.

pression of CHP reduced RasV12 increases in resting pH_i from 7.17 ± 0.02 to 7.01 ± 0.03 and Gα13Q226L increases in resting pH_i from 7.15 ± 0.02 to 7.00 ± 0.01. Ha-Ras and Gα13 couple to the activation of NHE1 through distinct signaling pathways; Ras action is mediated by Raf-1 and MAP kinase kinase (MEK), while Gα13 acts through independent pathways mediated by Cdc42 and RhoA (15). Stimulation of NHE1 by constitutively activated Cdc42V12 and RhoAV14 was also inhibited by overexpression of CHP (Fig. 3*B*).

Immunoprecipitation of Myc-tagged CHP from ^{32}P-orthophosphate-labeled cell lysates indicated that CHP is phosphorylated *in vivo*. CHP phosphorylation was greatest in quiescent cells maintained in serum-free medium for 18–24 h (Fig. 4*A*, lane 2) and was decreased with the addition of serum (10%, for 15 min; Fig. 4*A*, lane 3). CHP phosphorylation was also reduced by the expression of RasV12, Gα13Q226L, Cdc42V12, and RhoAV14, but not by the expression of Gα_sQ227L, which does not couple to the regulation of NHE1 activity (refs. 16 and 38; Fig. 4*B*). The functional association of CHP–NHE1 may therefore depend on the phosphorylation state of CHP, and conditions that stimulate NHE1 activity may decrease the phosphorylation of CHP. Additionally, divergent GTPase-mediated mechanisms may converge to activate NHE1 through the regulation of CHP phosphorylation.

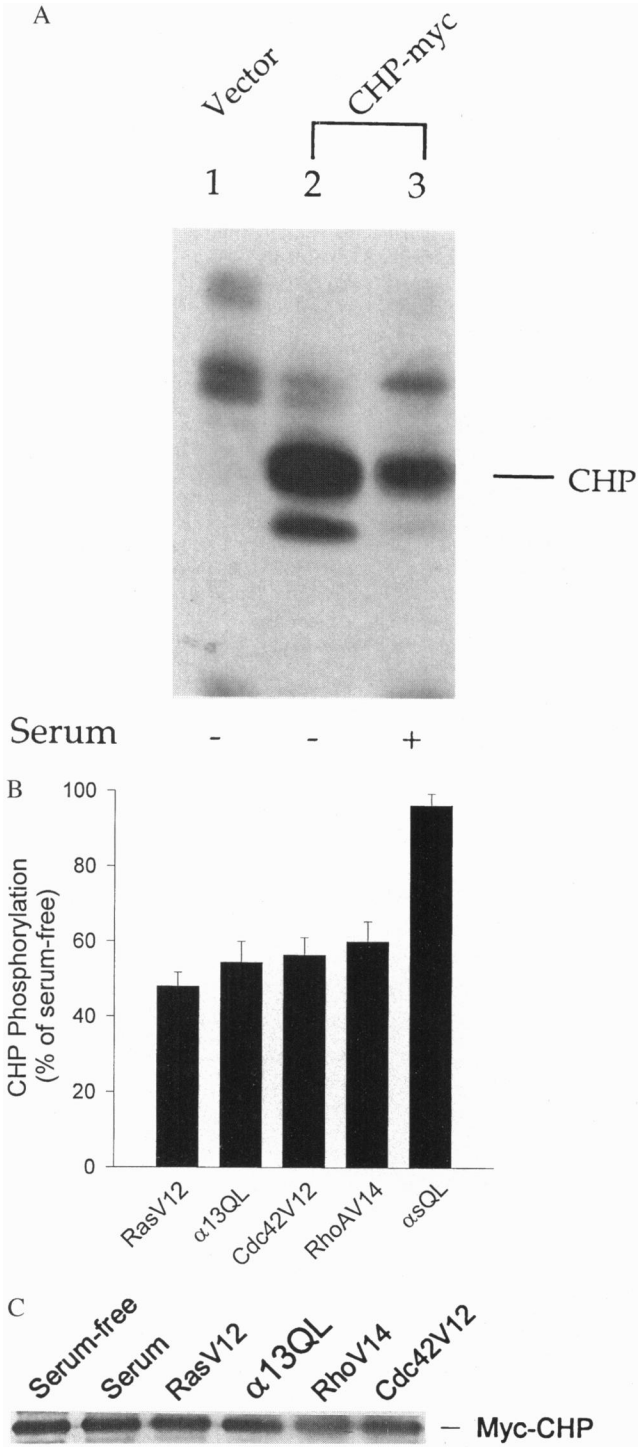

FIG. 4. Phosphorylation of CHP *in vivo*. (*A*) Myc-tagged CHP was immunoprecipitated from lysates prepared from [^{32}P]orthophosphate-labeled CCL39 cells transfected with empty vector (lane 1), or CHP–Myc-maintained in serum-free medium (lane 2), or supplemented with serum for 15 min (lane 3). (*B*) Effects of mutationally activated GTPases on CHP phosphorylation. Data are presented as the percentage of maximal phosphorylation observed in serum-deprived quiescent cells and represent the means ± SEM of three separate transfections. (*C*) Immunoblots probed with Myc antibodies demonstrate that equivalent amounts of Myc-tagged CHP were immunoprecipitated.

We have identified a ubiquitously expressed protein, CHP, that specifically interacts with NHE1 *in vitro* and *in vivo*. CHP binds to the regulatory site on NHE1 (amino acids 567–637) that has been demonstrated to be critical for growth factor activation of the exchanger. Deletion of this CHP-binding region completely abolishes growth factor-stimulated exchange activity (37), suggesting that CHP may regulate mitogenic activation of NHE1. Overexpressed CHP has no effect on the quiescent exchanger, but it inhibits serum- and GTPase-stimulated NHE1 activity, suggesting that CHP may constitutively bind to the exchanger in the resting state and be released upon growth factor stimulation. Release of CHP could trigger the exchanger to adopt an activating conformation, or it could allow binding of an NHE1 positive regulator. Overexpression of CHP in cells probably overrides the dissociation of endogenous CHP from NHE1 upon stimulation, and this constitutive CHP-NHE1 binding blocks NHE1 activation. The observation that CHP is a phosphoprotein and that a decrease in its phosphorylation is associated with an increase in exchange activity suggests that changes in the CHP phosphorylation state may regulate its binding to NHE1 and, in turn, its action on exchange activity. Our findings suggest that CHP plays a critical role in regulating mitogenic stimulation of Na-H exchange.

During the preparation of this manuscript, a novel protein, p22, was identified by Barroso *et al.* (35). p22 belongs to the EF-hand superfamily of Ca^{2+}-binding proteins and may be required for constitutive exocytic membrane traffic. Based on the sequence identity, we speculate that p22 is the CHP homologue in rat. The ability of CHP and p22 to regulate NHE1 activity and vesicular fusion suggests that these proteins may have multiple actions, similar to their homologous Ca^{2+}-binding proteins calcineurin (39) and CaM (40). Alternatively, the binding and regulation of NHE1 by CHP may involve the process of exchanger trafficking. Although it is currently unknown whether the Na-H transporter is regulated by recruitment to the plasma membrane from a vesicular pool, GLUT4, a glucose transporter, is sequestered in secretary vesicles and undergoes an activation-induced translocation to the plasma membrane (41). Further studies will determine the physiological role of CHP in regulating NHE1 activity and vesicular fusion.

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