Calcineurin Homologous Protein as an Essential Cofactor for Na\(^+\)/H\(^+\) Exchangers*  

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The Na\(^+\)/H\(^+\) exchangers (NHEs) comprise a family of transporters that catalyze cell functions such as regulation of the pH and volume of a cell and epithelial absorption of Na\(^+\) and bicarbonate. Ubiquitous calcineurin B homologous protein (CHP or p22) is co-localized and co-immunoprecipitated with expressed NHE1, NHE2, or NHE3 independently of its myristoylation and Ca\(^{2+}\) binding, and its binding site was identified as the juxtamembrane region within the carboxyl-terminal cytoplasmic domain of exchangers. CHP binding-defective mutations of NHE1–3 or CHP deletion by injection of the competitive CHP-binding region of NHE1 into Xenopus oocytes resulted in a dramatic reduction (>90%) in the Na\(^+\)/H\(^+\) exchange activity. The data suggest that CHP serves as an essential cofactor, which supports the physiological activity of NHE family members.

The Na\(^+\)/H\(^+\) exchanger (NHE) is an electroneutral plasma membrane transporter that catalyzes H\(^+\)-extrusion coupled to Na\(^+\)-influx (1, 2). Six identified NHE isoforms exhibit different tissue expression patterns (1–4): NHE1, in all tissues; NHE2–4, mostly in epithelial cells; NHE5, in brain; and NHE6, in mitochondria. These isoforms seem to have distinctive properties despite their overall structural similarity. For example, NHE1 and NHE3, which have been the most intensively studied isoforms, are involved in regulation of intracellular pH and cell volume in all cell types and Na\(^+\) and bicarbonate absorption in the epithelial cells, respectively. These two isoforms exhibit very different modes of regulation by many physiological factors as well as a large difference in the sensitivity to inhibitors such as amiloride derivatives (1, 2). Furthermore, targeted gene disruption of NHE1, NHE2, or NHE3 produces remarkably different phenotypes in mice; epilepsy and seizure for NHE1, reduced acid secretion in stomach for NHE2, and reduced salt absorption in kidney and low blood pressure for NHE3, respectively (5–7). The functional diversity may suggest that NHE isoforms have fundamental differences in the regulatory mechanism.

All NHE molecules comprise two major domains, amino-terminal transmembrane (~500 amino acids) and carboxyl-terminal cytoplasmic domains (~300 amino acids). The latter has been suggested to function as a regulatory domain involving multiple accessory factors (1, 2). For example, calmodulin (8, 9) and the NHE3 regulatory factor (10) have been suggested to regulate NHE1 and NHE3 by interacting with their cytoplasmic domains, respectively. Five years ago, Lin and Barber (11) identified a novel Ca\(^{2+}\)-binding protein CHP that interacts with NHE1 and exerts regulatory influences on its activity. CHP is ubiquitously expressed and homologous to the calcineurin B subunit (11). This same protein has been identified independently as a factor (called p22) required for the vesicular transport of proteins (12). More recently, this protein has been reported to inhibit the calcineurin phosphatase activity (13) or to associate with microtubules (14). Lin and Barber (11) reported that overexpression of CHP with a tag for detection in CCL39 fibroblastic cells inhibits the activation of NHE1 induced by serum or small G proteins and suggested that CHP may be dissociated from the exchanger upon mitogenic stimulation (11). However, the role of endogenous CHP in NHE1 has not yet been clarified. Our preliminary experiment showed that CCL39 cells express a significant amount of endogenous CHP that may be sufficient to form a complex with endogenous NHE1 at a 1:1 molar ratio. Therefore, the interpretation of the reported effect of CHP overexpression in NHE1 function (11) may not be straightforward. Clearly, to assign the definitive role to CHP, it is necessary to compare the exchanger functions in the presence or absence of bound CHP.

In this study, we have characterized the role of endogenous CHP in the functions of NHE1 and other NHE isoforms not determined previously. We show here that CHP is a key molecule that supports the activities of multiple exchanger isoforms. This is the first study to show that different exchangers require physical interaction with a common protein to express physiological activity.

MATERIALS AND METHODS

Cell Culture and cDNA Transfection—The exchanger-deficient PS120 cells (15) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 7.5% fetal calf serum at 37 °C in an atmosphere of 95% air and 5% CO\(_2\). Plasmids were transfected into PS120 cells (5 × 10⁵ cells/10-cm dish) by means of the calcium-phosphate coprecipitation technique. Cell populations that stably express NHE variants were selected by means of the repetitive H\(^-\)killing selection procedure (16). For stable expression of CHP-GFP fusion protein variants, single clones expressing proteins were isolated using GFP fluorescence as a marker after selection with G418.

Construction of Expression Vectors for NHE and CHP Variants—Plasmids carrying cDNAs for human NHE1, rat NHE2, and rat NHE3, and their mutants were all cloned into the mammalian expression vector pCE. All these constructs were produced by means of the polymerase chain reaction (PCR)-based strategy (16). The cDNA for CHP was obtained from human blood by reverse transcriptase-PCR based on the reported sequence (11). CHP-GFP fusion protein plasmids were constructed using vector pEGFP-N1 (Clontech) by means of the

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The abbreviations used are: NHE, Na\(^+\)/H\(^+\) exchanger; CHP, calcineurin B homologous protein; GFP, green fluorescent protein; MBP, maltose-binding protein; GST, glutathione S-transferase; PCR, polymerase chain reaction; NHS-biotin, N-hydroxysuccinimido-biotin; EIPA, ethylisopropylamiloride.
Role of CHP in NHE

**Expression and Purification of NHE and CHP Fusion Proteins**—MBP or GST fusion protein plasmids were constructed using vector pMAL-c (New England Biolabs) or pGEX-2TK (Amersham Pharmacia Biotech) by means of the PCR-based method. Proteins were purified according to the manufacturer's protocol. For production of recombinant CHP, the full CHP sequence with 6 His residues was cloned into pET11 (Stratagene). This plasmid was transformed into *Escherichia coli* cells (BL21) either alone or together with *pMAL-c* carrying cDNA for yeast N-myristoyltransferase (kindly provided by Dr. Gordon, Washington University). After protein induction, the transformed bacteria were sonicated and centrifuged. The pellet was suspended in the lysis buffer containing 6 M guanidine hydrochloride. CHP proteins were purified on a ProBondTM resin column (Invitrogen), according to the manufacturer's protocol. Proteins were renatured by dialysis overnight against 150 mM NaCl and 10 mM Hepes, Tris, pH 7.4. His-tagged NHE1 proteins were also purified from *E. coli* in a similar way.

**Immunoprecipitation, Immunoblotting, and Surface Labeling with NHS-Biotin**—Polyclonal anti-NHE3 or anti-CHP antibodies were produced by immunizing rabbits with the MBP-NHE3-(470–831) or GST-CHP-(1–195) fusion proteins. Immunoprecipitation and immunoblotting were performed essentially as described previously (8, 16). Briefly, cells were solubilized with 1% Triton X-100 in 20 mM Hepes, Tris, pH 7.4, 150 mM NaCl, and protease inhibitors, and the cell lysate was incubated with the antibodies indicated in the figure as described under “Materials and Methods.” The proteins were eluted from resin with 50 mM maltose, pH 6.0, and then incubated for 1 h at 4 °C with 30 μl of amylose resin pretreated with the MBP-NHE1-(503–815) fusion protein (200 μg) in buffer (in mM: NaCl, 150; Hepes/Tris, pH 7.4, 10; and CaCl2, 1; or EDTA, 1). After washing, the proteins were eluted from resin with 50 mM maltose, electrophoresed, and then visualized by Coomassie Brilliant Blue staining as described under “Materials and Methods.” Control, no pretreatment with the MBP-NHE1 protein.

**PCR-based method.** Inserted DNA fragments containing mutations were confirmed by sequencing plasmids with a DNA sequencer model 377 (ABI) to ensure the fidelity of construction.

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**FIG. 2. Properties of NHE mutants.**

- **a.** amino acid sequence alignment of the CHP-binding domain of NHEs and the mutant constructs. The CHP binding domains of mammalian NHE1–5, *Xenopus* NHE, and the mutant constructs were aligned. Hydrophobic amino acids are underlined.
- **b.** expression level of various NHE variants. Lysates (20 μg) of cells expressing the mutant exchangers shown in **a** were analyzed by immunoblotting with anti-NHE1 (left) or anti-NHE3 (right) antibody. c, surface labeling of the NHE1 or NHE3 protein was carried out using NHS-biotin, as described under "Materials and Methods." d, lysates of cells expressing various NHE variants were subjected to co-immunoprecipitation experiments with anti-CHP antibody followed by immunoblotting with anti-NHE1 or anti-NHE3 antibody. e, and f, the extents of surface labeling of NHE1 variant proteins with NHS-biotin as in c and the amounts of them recovered on immunoprecipitation with anti-CHP antibody as in e, normalized as to the expression level of the high molecular weight NHE1 protein. The data represent means ± S.D. (n = 3), relative to the values for wild-type NHE1 (WT). g, the rates of EIPA-sensitive 22Na uptake by NHE1 variants were measured using cells pH i-clamped at 5.6. Inset, data for NHE2 and NHE3 and their mutants. Data are means ± S.D. (n = 3).

**RESULTS AND DISCUSSION**

**Identification and Characterization of the CHP-binding Domain in NHEs—**CHP is a myristoylated protein with four Ca2+-binding motifs (EF-hands) (Refs. 11, 12; Fig. 1a). CHP is expressed in virtually all cells (11) including the exchanger-deficient PS120 cells used in our expression study (Fig. 1b). Co-immunoprecipitation studies involving PS120 cells stably expressing NHE1 revealed that CHP is tightly associated with NHE1 (Fig. 1b). To identify the CHP-binding domain in NHE1, we produced a series of MBP fusion proteins containing various regions of the carboxyl-terminal cytoplasmic domain (amino acids 503–815) of NHE1 (see Fig. 1a) and examined their interaction with GST-CHP fusion proteins by far-Western analysis (Fig. 1, c and d). The analysis revealed that amino acids 515–530 of NHE1 are required for CHP-binding (Fig. 1, c and d), which was further confirmed by co-immunoprecipitation experiments involving cells expressing several deletion mutants of NHE1 (Fig. 1c). The identified CHP-binding domain is different from the previously reported one (amino acids 567–635; Ref. 11). The sequence of the CHP-binding domain was found to be well conserved among mammalian NHE isoforms NHE1–5 (see Fig. 2a), suggesting that all these isoforms may interact with CHP. In fact, CHP bound to NHE3 (see Fig. 2d).

**Effects of Mutation within CHP-binding Domains of NHEs—**The CHP-binding domain of NHE1 is predicted to form a helical wheel diagram for amino acids 518–535 of NHE1. The sequence of the CHP-binding domain was different from the previously reported one (amino acids 567–635; Ref. 11). The identified CHP-binding domain is shown for amino acids 518–535 of NHE1. To identify the CHP-binding domain in NHE1, we produced a series of MBP fusion proteins containing various regions of the carboxyl-terminal cytoplasmic domain (amino acids 503–600) of NHE1(N2) and NHE1(N4), in which amino acids 503–600 of NHE1 were replaced by the corresponding region of NHE2 or NHE4, respectively (Fig. 1c). CHP also bound to these exchangers (Fig. 1f). Thus, NHE1–4 all contain CHP-binding sites in the juxtamembrane region of the carboxyl-terminal cytoplasmic domain.

We have produced His-tagged recombinant CHP proteins with and without a myristoyl moiety using an *E. coli* expression system. Both CHP proteins bound to NHE1 in the absence or presence of Ca2+, as revealed in the pull-down assay with MBP-NHE1 fusion proteins (Fig. 1g). Furthermore, we produced two chimeric exchangers, NHE1(N2) and NHE1(N4), in which amino acids 503–600 of NHE1 were replaced by the corresponding region of NHE2 or NHE4, respectively (Fig. 1c). CHP also bound to these exchangers (Fig. 1f). Thus, NHE1–4 all contain CHP-binding sites in the juxtamembrane region of the carboxyl-terminal cytoplasmic domain.

**Measurement of 22Na Uptake—**The rates of ethylisopropyl-amilo-ride (EIPA)-sensitive 22Na uptake by PS120 cells expressing NHE variants were measured using cells pH i-clamped at 5.6 by the K+/nigericin method (19). For measurement of 22Na uptake by oocytes, cells were preincubated for 1 h in NH4Cl medium (in mM: NH4Cl, 80; CaCl2, 1; MgCl2, 1; and Hepes/Tris, pH 7.4, 10), washed twice with choline-Cl medium (in mM: choline-Cl, 80; CaCl2, 1; MgCl2, 1; and Hepes/Tris, pH 7.4, 10) and then incubated for 15 min in the same medium additionally containing 1 mM 22NaCl (370 kBq/ml), 1 mM ouabain, and 0 or 0.1 mM EIPA. Oocytes were washed four times with ice-cold, nonradioactive choline-Cl medium, and then 22Na radioactivity was counted.
Intriguingly, these mutations 4Q, 4R, 6Q, and 2Q/4R dramatically reduced the exchange activity, as measured as EIPA-sensitive \( ^{22}\text{Na}^+ \) uptake at an acidic intracellular pH of 5.6, to <10% of the level in the wild-type (Fig. 2g). A similar reduction in activity also occurred for deletion mutants D515–530 (not shown). As summarized in Fig. 2, f and g, the exchange activity changed in parallel with the CHP-binding ability for various NHE1 mutants, despite the fact that nearly the same amounts of proteins were expressed in the surface (Fig. 2e). These data suggest that CHP binding is required for optimal activity of NHE1. We could not further analyze regulatory or kinetic properties of CHP binding-defective mutant exchangers (4Q, 4R, 6Q, and 2Q/4R) because of their low activity (\( V_{\text{max}} \)). However, cells expressing a mutant (518Q/522Q) with low affinity for CHP but with modest activity show similar properties to the wild-type NHE1, i.e. an intracellular pH dependence of \( ^{22}\text{Na}^+ \) uptake with a pK of \( \sim 6.6 \) and cytoplasmic alkalinization by 0.1 pH unit in response to \( \alpha \)-thrombin, platelet-derived growth factor, phorbol ester, and hyperosmotic stress (data not shown). These properties probably reflect those of the CHP-bound but not CHP-unbound mutant exchanger because only the former exhibits measurable high activity as shown in oocyte experiments described below. Similar to NHE1 mutants, NHE2–3 or NHE3–4 mutants (see Fig. 2a) did not interact with CHP (see Figs. 2d and 4, h–i). In addition, these CHP binding-defective mutants of NHE2 and NHE3 exhibited markedly reduced exchange activity (Fig. 2g, inset).

**Injection of CHP-binding Region of NHE1 Dramatically Reduces the Activity of Xenopus Oocyte Exchanger**—The above data strongly suggest that CHP binding is required for the exchange activity of NHE1–3. To examine this further, we used a Xenopus oocytes expression system. Oocytes are known to have an endogenous NHE, whose amino acid sequence is similar to that of human NHE1 (overall homology, 78%; Refs. 24,
25. CHP-binding domains were well conserved in the two species (see Fig. 2a). Oocytes also have an endogenous CHP detectable on immunoblotting with anti-human CHP antibody (Fig. 3a). We produced His-tagged human NHE1 proteins (amino acids 503–600) containing the wild-type sequence or CHP binding-defective mutations (Fig. 3b). Injection of the His-tagged wild-type protein into oocytes, but not that of mutant proteins, resulted in the disappearance of Xenopus NHE1 from the CHP immunoprecipitates (Fig. 3c, middle). Furthermore, anti-CHP antibody co-precipitated the His-tagged wild-type protein with endogenous CHP, but not mutant proteins (Fig. 3c, bottom). These data indicate that the injected human CHP-binding protein depletes bound CHP from Xenopus NHE. Importantly, we found that injection of the His-tagged wild-type protein abolished the endogenous exchange activity in oocytes almost completely (Fig. 3d). A relatively long time (>3 h) was required for inhibition of the exchange activity after injection of proteins (Fig. 3e), suggesting that CHP is slowly released from Xenopus NHE. On the other hand, the exchange activity of human NHE1 expressed in oocytes was significantly increased when human CHP was co-expressed (Fig. 3f). These data also strongly suggest that CHP is required for high exchange activity.

Subcellular Localization of GFP-tagged CHP Proteins—CHP fusion protein conjugated with green fluorescent protein (CHP-GFP) was expressed uniformly in the cytosol of PS120 cells (Fig. 4c). This fusion protein became partly localized in the cell surface when exogenous NHE1 was co-expressed (Fig. 4d). However, CHP-GFP was not localized in the surface membrane when NHE1 mutant 4Q was expressed in the membrane (Fig. 4, b and c). Therefore, NHE1 seems to be the principal target for CHP in the membrane. We stably overexpressed two GFP-conjugated CHP mutant proteins (CHP2A-GFP and CHPET3-GFP) that lack the ability to be myristoylated or to bind Ca2+ (12). Consistent with the in vitro binding data (see Fig. 1g), these proteins were partly localized in the surface membrane together with the NHE1 protein (Fig. 4, f and g), suggesting that they might have replaced endogenous CHP. Because expression of these proteins did not inhibit 22Na+ uptake activity (Fig. 4f), it appears that neither myristoylation nor Ca2+ binding is essential for exchange activity. CHP-GFP was also partly localized in the surface membrane when it was co-expressed with NHE2 or NHE3, although a significant amount of CHP-GFP still remained in the cytosol or an intracellular compartment (Fig. 4, h and j). However, such surface expression was abolished by mutations (NHE2-3R and NHE2-4R) introduced into these NHE isoforms (Fig. 4, i and k), indicating that CHP binding was lost in these mutants.

It has generally been considered that a single polypeptide for each NHE isoform is sufficient for normal exchange activity. However, the present data provide evidence that NHE1 requires an extra cofactor protein, CHP, for it to express physiological activity. Our data also suggest the same role of CHP for NHE2-4. A potential CHP-binding motif also exists in NHE5 (Ref. 3; see Fig. 2a), but not in mitochondrial NHE6 (4). Based on these findings, we propose that CHP functions as an integral cofactor common to plasma membrane-type Na+/H+ exchangers.

Na+/H+ exchange presumably occurs through the transmembrane domain of an exchanger. Cells expressing a NHE1 mutant (Δ515) with the complete carboxyl-terminal cytoplasmic domain (amino acids 516–815) deleted still retain low exchange activity that can be detected by expressing a high copy number of a mutant molecule (16). This is consistent with the present finding that mutants lacking the CHP-binding site are able to exhibit low exchange activity (5–10% of the wild-type level). We previously showed that the exchange activity at physiological pH1 is markedly decreased by deletion of different regions in the amino terminus (subdomain I, amino acids 515–595) of the NHE1 cytoplasmic domain (19). These data suggest that subdomain I is essential for normal exchange activity. This domain with bound CHP would therefore function as a key structure that interacts with the transmembrane domain (see Fig. 1a), thereby permitting the ion translocation pathway to maintain its physiologically relevant active conformation. More detailed structural information including the crystal structure of the CHP-NHE complex is necessary to reveal how CHP is involved in this important task of subdomain I.

Subdomain I contains the CHP-binding region (amino acids 515–530) as well as two potential phosphatidylinositol 4,5-bisphosphate (PIP2) binding motifs (amino acids 509–516 and 552–560 for the human NHE1 sequence) identified by Aharonovitz et al. (26) who provided evidence that a reduction of cell PIP2 causes marked inhibition of the exchange activity of NHE1. Their study, however, provided little information regarding whether or not the effect of PIP2 is mediated by the above binding motifs in situ. In this study, we found that the injected His-tagged wild-type NHE1 protein (amino acids 503–600) almost completely abolished the exchange activity in oocytes, whereas mutant protein 4R lacking the ability to bind CHP did not (Fig. 3d). This finding seems to argue against the view that the function of CHP is directly related to PIP2, because the injected wild-type and mutant 4R proteins would not be much different in their PIP2 binding (see Fig. 2a).

We observed that the surface localization of CHP-GFP in NHE1 transfectants was not affected significantly by ionomycin, ATP depletion, phorbol ester, serum, or other growth factors (not shown), which is inconsistent with the previous proposal (11) that CHP may be dissociated from NHE1 upon growth factor activation. Whether or not the bound CHP is involved in acute regulation of the exchanger is not clear at present. Although CHP interacts with multiple NHE isoforms, it is still possible that the activity of the exchanger is regulated through post-translational modifications of the associated CHP in response to extracellular stimuli. Indeed, myristoylated CHP alters its conformation in a Ca2+-dependent manner (see Fig. 1g), similar to some other Ca2+-myristoyl switch proteins (20). It is possible that such a conformational change of bound CHP alters the structure of subdomain I and regulates the transport activity.

In conclusion, the present study revealed that multiple exchangers require physical interaction with a common protein, CHP for exchange activity. This function does not appear to require Ca2+ binding and myristoylation in CHP. To our knowledge, this is the first finding to show that a Ca2+-binding protein supports the activity as an integral component of the secondary active transporter.

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