Loss of calcineurin homologous protein-1 in chicken B lymphoma DT40 cells destabilizes Na\(^+\)/H\(^+\) exchanger isoform-1 protein

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Abstract

Calcineurin homologous protein-1 (CHP1), a Ca\(^{2+}\)-binding 22-kDa protein similar to a regulatory subunit of calcineurin, binds to the juxtanuclear region of NHE1–5 (5, 19, 34). A closely related isoform, CHP2 (77% similarity), is expressed in a limited range of tissues and cells, including the small and large intestines and some cancer cell lines (15, 35), and is also capable of interacting with NHE1–5. CHP1 is ubiquitously expressed and interacts with several proteins aside from NHE1–5, such as the kinases DRAK2 (18, 21), protein phosphatase calcineurin (20), and gyceraldehyde-3-phosphate dehydrogenase (1). Therefore, CHP1 is thought to be a multipotent regulatory protein. However, NHEs are important partners for CHP1 in cellular functions, because NHEs maintain intracellular ion environments, which affect virtually all biochemical reactions.

Although several groups report interactions between CHP1 and NHE1 (19, 34), the functional relationship between these proteins is controversial. Lin and Barber (19) reported that overexpression of CHP1 in CCL39 cells caused inhibition of NHE1 activation induced by serum or small G proteins. Pang et al. (34) demonstrated that PS120 cells expressing NHE1 incapable of interacting with CHP1 exhibited extensive loss of NHE1 activities. They also showed that injection of an excessive amount of peptides corresponding to the CHP-binding region in NHE1 inhibits NHE1 activities in Xenopus oocytes. Therefore, they concluded that CHP1 is an essential cofactor for the exchange activity of NHE1.

Because previous results were based largely on CHP1 overexpression and dominant-negative approaches, we took a loss-of-function approach to assess the function of CHP1 in relationship to NHE1. Here we establish CHP1-deficient cells by gene targeting in chicken B lymphoma DT40 cells (3, 48). We show that in CHP1-deficient cells, the cellular Na\(^+\)/H\(^+\) exchange activities were totally lost because of significant loss of NHE1 protein, which is potentially destabilized in the absence of CHP1. These observations suggest a novel activity of CHP1 relevant to NHE1.
MATERIALS AND METHODS

Cell culture. Chicken DT40 cells were kindly provided by Dr. Tomohiro Kuroasaki (RIKEN Research Center for Allergy and Immunology, Yokohama City, Kanagawa, Japan) and maintained in RPMI medium (R8758; Sigma-Aldrich, St. Louis, MO) containing 10% fetal calf serum, 1% chicken serum, and 80 μM 2-mercaptoethanol at 39°C in 5% CO₂ (3, 48). Opossum kidney OK cells (17) and human HEk293 cells were maintained in MEM medium (Sigma-Aldrich) containing 10% fetal calf serum at 37°C in 5% CO₂. HEk293 and OK cell lines were originally obtained from American Type Culture Collection.

Vector construction and gene targeting. The chicken CHPI fragment was amplified from a DT40 cDNA library with PCR using primers containing flanking EcoRI sites (ecggagtcATGGGTTC-CCGAGGCTCTAC and ecggagtcACGTTGAAATGCTACG) and cloned into pBluescriptII KS(+) vector. A CHPI expression vector (pApuro II-CHPI) was constructed by introducing the cloned CHPI fragment into the EcoRI site downstream of the actin promoter in the pApuro II vector (42). A chicken CHPI genomic clone was isolated from a DT40 genomic library using the entire CHPI cDNA as a probe. The isolated genomic clone contained exons 1–3. To construct the targeting vector, a 4.75-kbp fragment containing exon 3 was excised with HindIII and NotI and cloned into pBluescriptII KS(+) vector. Then, histidinol or benzylpenicillin resistance cassette was inserted into the BamHI site in exon 3. Thirty micrograms of NotI-linearized targeting vector were introduced into 10⁷ DT40 cells in 0.5 ml of ice-cold PBS in a 0.4-cm cuvette by electroporation at 550 V, 250 μF. After 10 min on ice, cells were suspended in 20 ml of growth medium and incubated for 24 h. Cells were then suspended in 40 ml of medium containing either 1 mg/ml histidinol or 50 mg/ml benzylpenicillin and dispensed to each well of 96-well culture plates. After 7–10 days, drug-resistant colonies were obtained. The drug-resistant cells were cultured, and gene targeting was confirmed by Southern blotting using the 0.55-kbp EcoRI fragment between exon 2 and exon 3. Southern blotting and other gene manipulations were performed according to published protocols (25, 39).

VECTOR CONSTRUCTION OF MUTANT CHPI. CHPI expression vectors containing mutant EF-hand motifs (E134A, E175A) were generated by site-specific mutagenesis using PCR with overlapping primers and pApuro II-CHPI as the template (39). Overlapping primers containing Glu-to-Val substitutions (GGGGTACCCCTCAGTGAAGAAATCGAAT and GGGGTCAGTGAAGAAATCGAAT) were synthesized by Applied Biosystems (Foster City, CA) and introduced into pApuro II vector, and the entire sequence was verified using an automated DNA sequencer ABI3100 (Applied Biosystems, Foster City, CA).

Analysis of NHE1 mRNA. Total RNA was isolated from 10⁷ DT40 cells with the Sepasol RNA isolation solution (Nacalai Tesque, Kyoto, Japan), following the manufacturer’s instruction. RT and PCR were performed with an RNA PCR kit (AMV, version 3.0) (Takara, Otsu, Japan). The primers were GTGCTGTGGCTCTCACCATACTCAG and GGATATCCATGGTTCCGAGGT (β-actin, and TCTCGGGGTCACTACGGGCAC and CTCTCTGGGCTCTACCCAAACC for NHE1). PCR was performed from serially diluted templates, and it was confirmed that the amplification was done efficiently and nonsaturated. Quantitative real-time PCR was performed using the 7300 Real Time PCR system (Applied Biosystems) and SYBR Premix Ex Taq (Takara) according to the manufacturer’s instructions. The primers were GAGAAATTGTGCGTGGACATCA and CCTGGAACCTTCTCATGCGCCA for the β-actin gene (product size, 152 bp), and TGTCACCGGACCGGAGATGAGA and TACGGTTCTGGCAACTGGAGTCT for the NHE1 gene (product size, 184 bp). Quantification with a standard curve was performed using the software provided with the real-time PCR system. The data were normalized with respect to the expression of β-actin. Specific amplification was confirmed by referring to the dissociation curve and by performing electrophoresis.

Immunoblotting and surface labeling. DT40 cells were washed with PBS, pelleted by centrifugation, and lysed in PBS containing 1% NP-40, 1% Triton X and 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin. Cells were disrupted by sonication in an ice-cold bath and mixed with SDS-PAGE sample buffer. To avoid NHE1 aggregation, boiling or heat treatment was not performed. Samples were resolved by SDS-PAGE and transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA) at 180 mA for 90 min in transfer buffer (32 mM glycine, 124 mM Tris, 0.01% SDS, and 20% methanol) with a wet tank blotting apparatus. The blotted membrane was incubated in PBS containing 0.01% Tween 20 and 0.1% SDS for 30 min and washed twice with PBST (PBS containing 0.1% Tween 20). The immunoreaction was performed with Can Get Signal solution (Toyobo, Osaka, Japan), following the manufacturer’s instructions. Signals were detected with chemiluminescence using enhanced chemiluminescence (ECL; GE Healthcare, Chalfont St. Giles, UK), and images were acquired with Hyperfilm ECL (GE Healthcare) or a cooled charge-coupled device (CCD) imaging system (LAS-1000; Fuji Film, Ashigara, Japan). For surface labeling experiments, DT40 cells (10⁷ cells) were washed with PBS(−) (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) and incubated with 2 mg/ml EZ-Link NHS-SS-biotin (Pierce Biotechnology, Rockford, IL) in 1.5 ml of PBS(−) at 4°C for 1 h. The cells were pelleted and suspended in 0.1 M glycine-PBS(−), washed with PBS(−), and lysed in PBS containing 1% NP-40, 1% Triton X and 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin. Debris was removed by centrifugation at 20,000 g for 10 min. The lysate was incubated with NeutrAvidin beads (immobilized NeutrAvidin on agarose, Pierce) for 30 min. Beads were washed twice with PBS(−), and proteins were eluted in buffer with 50 mM DTT. NHE1 antibodies were purchased from Becton Dickinson and Chemicon. Antibodies for CHPI and CHP2 were generated as described previously (15, 21). Antibodies for Na⁺/K⁺ ATPase α-1 and α2 were purchased from Upstate (Billerica, MA) and Chemicon (Temecula, CA), respectively.

Na⁺ uptake assay. Ethylisopropyl-amiloride (EIPA)-sensitive Na⁺ uptake was performed using the silicon layer method (44). DT40 cells (10⁷) were washed with isotonic choline chloride solution (125 mM choline chloride, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4). The cells were spun down, and the buffer was discarded; then cells were incubated for 1 h in isotonic NH₄Cl solution (50 mM NH₄Cl, 70 mM choline chloride, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4). Cells were washed twice with PBS(−), and proteins were eluted in buffer with 50 mM DTT. NHE1 antibodies were purchased from Becton Dickinson and Chemicon. Antibodies for CHPI and CHP2 were generated as described previously (15, 21). Antibodies for Na⁺/K⁺ ATPase α-1 and α2 were purchased from Upstate (Billerica, MA) and Chemicon (Temecula, CA), respectively.

Overexpression of NHE1. The FLAG-tagged NHE1 expression plasmid was constructed by introducing an EcoRI-SphI fragment containing rat NHE1 and a SphI-SalI fragment coding for FLAG tag and a stop codon into the pApuro II vector. The expression vector was
introduced into wildtype or CHP1-deficient DT40 cells by electroporation as described above. After 7–10 days, drug-resistant colonies were obtained. The drug-resistant cells were cultured, and expression of NHE1 was studied by immunoblot analysis.

Proteasome inhibition and immunoprecipitation. FLAG-tagged NHE1-expressing cells (CHP1+/+ and CHP1−/−) and wildtype cells (5 ml of 0.15 × 10⁶ cells/ml culture) were grown overnight and incubated for 3 h with or without 25 μM MG132 (Sigma-Aldrich) before lysis. The lysate was prepared as described above and then incubated with FLAG-M2 agarose beads (Sigma-Aldrich) for 30 min at 4°C. The beads were washed three times with PBS(−) and mixed with SDS-PAGE sample buffer. To avoid aggregation of NHE1, heat denaturation was not performed. The samples containing beads were applied to the wells of an SDS-PAGE gel. For detection of ubiquitinated proteins, polyclonal anti-ubiquitin antibody (Sigma-Aldrich and Stressgen) was used.

Knockdown of CHP1 in HeLa cells. Knockdown experiments were performed with Stealth siRNA (Invitrogen). siRNA and control RNA were designed using the manufacturer’s software (Invitrogen). The sense sequences of siRNA and control RNA were GGAAUCAUGC-GAACUUUGGCUCAUU and GGAAUCGCCAUUUCGGCUUAAUU, respectively. The cells (2 × 10⁶ cells) were plated in 2 ml of medium and transfected with RNA duplex (250 pm) in the presence of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 48 h, cells were lysed and subjected to immunoblot analysis.

RESULTS

Establishment of CHP1-deficient DT40 cells. To obtain CHP1-deficient DT40 cells, we disrupted the CHP1 locus by replacing the coding region of the gene with drug resistance cassettes. To generate the CHP1 deletion construct, we identified a 4.75-kbp region containing exon 3 from a DT40 cell genomic library and cloned it into a targeting vector; then a histidinol or blasticidin resistance cassette was inserted into the BamH1 site in exon 3 (Fig. 1A). We first isolated histidinol-resistant DT40 clones by transfection of the histidinol-resistant targeting vector. Subsequently, blasticidin-resistant clones were isolated by transfection of the second vector. Disruption of each allele was confirmed by Southern blotting (Fig. 1B).

For wildtype cells (Fig. 1B; lane WT), KpnI-digested fragments were probed with a CHP1 cDNA probe, and a wildtype fragment was observed at 13.6 kbp. In heterozygous CHP1-deficient cells (Fig. 1B; +/−), a 17.1-kbp fragment from the allele containing the histidinol resistance cassette (Fig. 1A) and a 13.6-kbp wildtype fragment were detected, whereas in homozygous cells, only the larger fragment was detected. The larger fragments were likely a mixture of histidinol and blasticidin gene-containing fragments, because of their similar sizes (17.1 and 16.4 kbp, respectively). Loss of CHP1 protein expression was confirmed by immunoblotting, using a CHP1-specific antibody (Fig. 2; WT, +/−, −/−). For additional analysis, we established rescued cell lines in which an expression vector containing a puromycin resistance cassette and CHP1 driven by the chicken actin promoter was introduced into homozygous CHP1-deficient cells. Expression of CHP1 was analyzed with the CHP1 antibody after isolating puromycin-resistant cells. Although some variation in expression levels was observed, rescue of CHP1 expression was detected [Fig. 1C; (−/−)/+[ lanes 1–3].

Next, we analyzed expression of CHP2 (15, 35), a closely related isoform of CHP1, in wildtype and CHP1-deficient...
DT40 cells (Fig. 2). In opossum kidney OK cells, which were used as a control, CHP2 was detected as described previously with a CHP2-specific antibody (Fig. 2) (15). In DT40 cells, CHP2 was not detected in wildtype or hetero- or homozygous CHP1-deficient cells (Fig. 2; +/− and −/−), nor was it detected in the rescued cells. These data showed that CHP2 expression was not induced by loss of CHP1.

**Na+/H+ exchange activities in CHP-deficient DT40 cells.**

Previous studies showed that NHE1 is a major isoform in blood cells (37, 38). We determined the IC_{50} of the NHE1-selective inhibitor EIPA for DT40 NHE as 3.2 × 10^{-8} M (data not shown). This pharmacological property was consistent with NHE1 (30) rather than other NHEs. The major physiological role of NHE1 is to protect against intracellular acidification by extrusion of H^+ in exchange for Na^+ (7). To assess the effect of CHP1 on NHE1 exchange activity, we used the NHE1-selective inhibitor EIPA and analyzed EIPA-sensitive ^{22}Na^+ uptake at an acidic intracellular pH (45). Intracellular acidification was induced by incubation in NH_4Cl-containing buffer and subsequent removal of NH_4Cl. Na^+ uptake was measured in the presence or absence of EIPA (Fig. 3A), and then EIPA-sensitive activity was calculated (Fig. 3B). Na^+ uptake in the presence of EIPA, which was from non-NHE1 activity, did not show significant differences among wildtype, CHP1-deficient, and rescued cells (Fig. 3A). In wildtype cells, 3.9 ± 0.3 nmol·mg^{-1}·min^{-1} EIPA-sensitive Na^+ uptake was observed (Fig. 3B; WT), and an extensive decrease in EIPA-sensitive Na^+ uptake (0.6 ± 0.5 nmol·mg^{-1}·min^{-1}) was observed for CHP1-deficient cells (Fig. 3B; −/−). In the rescued cell lines, Na^+ uptake was recovered to 3.7 ± 0.2 nmol·mg^{-1}·min^{-1} [Fig. 3B; (+/−)/+]. These results strongly suggest that CHP1 plays an important role in cellular Na^+/H^+ exchange activity mediated by NHE1.

**Expression of NHE1 in CHP-deficient DT40 cells.** Given the lower antiporter activity observed in CHP1-deficient cells, we evaluated expression levels of NHE1. RT-PCR was performed with RNA isolated from wildtype, CHP1-deficient, and CHP1-rescued DT40 cells (Fig. 4A). RT-PCR with specific primers showed that similar levels of NHE1 mRNA were expressed in all examined cell lines (Fig. 4A). These observations were confirmed under conditions in which amplification was not saturated using serially diluted templates (data not shown). We also performed quantitative real-time PCR with RNA isolated from wildtype and CHP1-deficient cells (Fig. 4B). The primers for real-time PCR were redesigned for efficient amplification. The mRNA level of NHE1 normalized to that of β-actin showed no significant difference between the wildtype and CHP1-deficient cells.

We next analyzed NHE1 protein expression with immunoblotting (Fig. 5). Typical large (90–110 kDa, highly glycosylated form) and small (60–80 kDa, core-glycosylated form) forms of NHE1 protein were detected, similar to that observed in mammalian cells, as described previously (Fig. 5A) (8). Surprisingly, a significant decrease (to ~8%) in NHE1 protein levels was observed in CHP1-deficient cells (Fig. 5C; −/−). In the CHP1-rescued cells, NHE1 protein levels recovered to 66% of that of wildtype cells (Fig. 5C). The expression level of NHE1 in rescued cells was not proportional to CHP1 expression levels. We also examined expression of NHE1 at the plasma membrane using a surface biotin labeling method (Fig. 5, B and D). Surface-expressed NHE1 tends to show a somewhat broad band in immunoblotting, possibly because of glycosylation. Levels of surface-expressed NHE1 in CHP1-deficient cells also exhibited a significant decrease (Fig. 5D). Moreover, recovery in the rescued cells was observed [Fig. 5, C and D; (+/−)/+]. These results suggest that CHP1 is required for NHE1 protein stabilization during the early biogenesis steps of NHE1 and for its resulting translocation to the plasma membrane. We suspected that the loss of NHE1 protein was possibly the result of improper and/or insufficient posttranslational modification of NHE1 in CHP1-deficient cells. Because of the difficulty in analyzing the low levels of NHE1 protein in CHP1-deficient cells, we forcibly overexpressed it by introducing an expression vector containing a puromycin resistance cassette and FLAG-tagged NHE1 driven by the chicken actin promoter.
into wildtype cells and CHPI-deficient cells. In NHE1-overexpressing cells, NHE1 was expressed typically >20-fold (data not shown). In the wildtype cells overexpressing NHE1, a slow-migrating, highly glycosylated form (Fig. 6A; H) and fast-migrating, core-glycosylated form (Fig. 6A; L) of NHE1 were observed. However, the slow-migrating form (H) was remarkably reduced in CHPI-deficient cells overexpressing NHE1, suggesting that, under overexpression conditions, NHE1 without CHPI is likely to be less sensitive to modification during the proper maturation steps.

To test whether proteasomal degradation was involved in the loss of NHE1 protein, we assessed NHE1 ubiquitination using the proteasomal inhibitor MG132 (Fig. 6B). The anti-NHE1 antibody performed poorly in immunoprecipitation. Thus we used FLAG-tagged NHE1-expressing cells for the efficient immunoprecipitation of NHE1. After MG132 treatment for 3 h, the cells were lysed, and immunoprecipitation was performed with anti-FLAG antibody-conjugated beads. Immunoblot analysis with anti-FLAG antibody revealed the two normal NHE1 species (H and L) and some cross-reactive bands. In addition, a very high molecular mass smear that stretched to the top of the gel was observed in MG132-treated cells (Fig. 6B; lane 2, 4 VH), suggesting that ubiquitinated species of NHE1 of very high molecular mass were accumulated under these conditions. Considering that the transfer efficiency of very high molecular mass proteins to membranes during immunoblot analysis is very low, the total amount of detected NHE1 species should not be decreased, while the amount of normal species of NHE1 (H and L) seemed to be decreased. Although normal species (H and L) of NHE1 were effectively immunoprecipitated (Fig. 6B; lanes 7–10), the immunoprecipitation of very high molecular mass species was inefficient in comparison (Fig. 6B; lanes 8 and 10, VH), probably reflecting their weaker accessibility to antibody during immunoprecipitation. The ubiquitinated protein signal in the immunoprecipitate was very weak (Fig. 6B; bottom), possibly because of the small amount of very high molecular mass proteins in the immunoprecipitate as mentioned above. However, ubiquitinated species were detected at higher levels in immunoprecipitates from MG132-treated CHPI-deficient cells (Fig. 6B; bottom, lane 10). These data suggest that NHE1 is a substrate of the ubiquitin-
proteasome system, and that degradation by the ubiquitin-proteasome system contributes to loss of NHE1 in CHP1-deficient cells.

We also tested the effect of proteasomal inhibitor degradation of endogenous NHE1. Short treatment as described above did not affect endogenous NHE1 (data not shown). Thus we treated the cells for 15 h. Although no effects were observed in wildtype cells, 15 h of treatment with MG132 or with another proteasomal inhibitor, MG101, resulted in an increase in the high molecular mass form of NHE1 protein in CHP 1-deficient cells (Fig. 7A; H) Very high molecular mass smears, as observed in cells overexpressing NHE1, were not observed. The restoration of high molecular mass NHE1 in inhibitor-treated CHP-deficient cells suggested that the high molecular mass NHE1 protein is the putative substrate for degradation in CHP1-deficient cells. The total amount of NHE1 protein (H+L) in the inhibitor-treated cells was not increased significantly by proteasomal inhibitor treatment. Other degradation pathways or mechanisms that attenuate NHE1 synthesis may also be involved in the loss of NHE1.

Effect of Ca\(^{2+}\) binding and myristoylation of CHP1 on NHE1. Rescue of CHP1 in CHP1-deficient cells caused recovery of NHE1 protein (Fig. 5). Previous studies revealed that CHP1 has a myristoylation site and two Ca\(^{2+}\)-binding sites (33, 43). We constructed mutant forms of CHP1 lacking either the myristoylation site or calcium-binding capability. To construct the myristoylation-deficient mutant, a glycine residue known to be myristoylated was substituted by alanine (G2A construct). To construct calcium binding-deficient CHP1, glutamate residues E134 and E175, which are essential for calcium binding in EF-hand motifs, were substituted by alanine residues at one (E134A and E175A constructs) or both (E134/175A construct) sites. Expression vectors harboring the puromycin resistance gene together with wildtype CHP1 or CHP1 with the amino acid substitutions were introduced into CHP1-deficient cells, and, after isolating puromycin-resistant clones, NHE1 expression was analyzed. In SDS-PAGE with non-heated samples, wildtype CHP1 migrated slightly faster than the calculated molecular mass (22 kDa), as reported previously (34). However, calcium binding-deficient mutants (E134A, E175A, and E134/175A) migrated at a slower rate than wildtype CHP1 (Fig. 7A), confirming that the mutations were present in CHP1. Because of random integration into the chromosome of transfected cells, CHP1 expression levels varied. However, partial recovery of NHE1 expression was observed in all mutant CHP1-expressing cells (Fig. 7A), suggesting that proper expression of NHE1 protein does not necessarily require calcium-binding activity or myristoylation of CHP1.

We also measured EIPA-sensitive Na\(^+\) uptake in two clones expressing mutant forms of CHP1. Cells expressing any mutant form of CHP1 showed a decrease in EIPA-sensitive Na\(^+\) uptake compared with wildtype cells (Fig. 7B). However, it should be noted that none of the mutant forms of CHP1 completely abolished NHE1 expression or activity. These results suggest that Ca\(^{2+}\) binding or myristoylation is not essential but is required for the NHE1 exchange activities in intact cells.

Effect of CHP1 knockdown on NHE1 in HeLa cells. To test whether the decrease of NHE1 in the absence of CHP1 was a specific event in DT40 cells, we performed knockdown experiments in human HeLa cells by transfecting them with CHP1 siRNA. CHP1 protein was decreased typically to ~10% of the normal level after 48-h posttransfection (Fig. 8A). The cells...
transfected with siRNA showed a decrease (~25%) in the level of NHE1 protein (Fig. 8, A and B). The decrease in NHE1 was moderate compared with the decrease in CHP1-deficient DT40 cells. Residual CH1 in knockdown cells may contribute to the stabilization of NHE1. These results are consistent with those obtained in CHP1-deficient DT40 cells and suggest that CH1 plays a ubiquitous role in stabilizing NHE1.

**DISCUSSION**

It was reported previously that CH1 plays a role in NHE1 activity as either an inhibitory (19) or an activating factor (34). Our report of Na\(^+\)/H\(^+\) exchange activities in chicken DT40 CH1 knockout cells supports the notion that CH1 plays an essential role as a cofactor to promote NHE1 activity (Fig. 3). However, we unexpectedly observed that CH1 depletion results in significant reduction in NHE1 protein (Fig. 5). Since NHE1 mRNA levels are not decreased in these cells (Fig. 4), loss of NHE1 protein is likely caused by posttranslational processes. These results indicate that CH1 is an essential cofactor of NHE1, since CH1 is required for the proper expression and destination to the cytoplasmic membrane.

NHE1 protein levels may be reduced in CHP1-deficient cells because of proteolysis. A recent study reported that the heterogeneous expression of mammalian NHE1 in yeast plasma membrane is stabilized in a strain with decreased levels of ubiquitin ligases (12). Our data suggest that NHE1 is a substrate of the ubiquitin-proteasome system and that degradation by the ubiquitin-proteasome system contributes to the loss of NHE1 in CHP1-deficient cells (Fig. 6, B and C). However, our data do not necessarily exclude the involvement of other mechanisms, such as attenuation of translation. Multiple mechanisms, including proteasomal degradation, may be involved in the loss of NHE1. A full understanding of the mechanism of NHE1 loss will require further studies.

A recent structural study of CH1 revealed that CH1 has a hydrophobic pocket that provides an NHE1-binding site (26). Binding of CH1 through this pocket may stabilize the NHE1 structure, allowing it to reach the plasma membrane. The CHP1-binding site is located at the juxtamembrane region in the hydrophilic domain of NHE1 (34). For the yeast plasma membrane NHE (Nha1p), the juxtamembrane region also contributes to proper expression in the plasma membrane (23). Although the sequences of animal and yeast NHEs are not homologous in this region, these observations suggest the structural importance of the juxtamembrane region of eukaryotic NHEs for their proper expression and activity.

In previous studies, Pang et al. (34) showed that overexpressed NHE1 mutants, which are incapable of interacting with CH1, are transported to the plasma membrane, leading to the conclusion that CH1 is not required for trafficking to the plasma membrane. Their results seem to be inconsistent with our results. If high levels of NHE1 are expressed from a transgene, a portion of the NHE1 would be expected to leak and escape degradation. This notion is supported by the similar levels of NHE1 protein in NHE1-overexpressing CHP1-deficient cells and NHE1-overexpressing wildtype cells (Fig. 6A). Our results indicate that the primary physiological function of CH1 is to stabilize NHE1 so that it can reach the plasma membranes. The difference in the modification of NHE1 between wildtype and CHP1-deficient cells under NHE1-overexpressing conditions (Fig. 6) suggests that NHE1 stability may be affected by impaired modifications caused by incorrect NHE1 conformations. The mechanism that is responsible for the impaired modification of NHE1 is currently unclear. It has been reported that impaired modification is associated with
cellular stress and proteasome degradation (49, 50). Such conformations and/or impaired modifications could contribute to stress control in the endoplasmic reticulum and subsequent degradation of NHE1, although determining the precise mechanism will require further study.

Lin and Barber (19) reported that overexpression of CHP1 causes inhibition of NHE1 activation induced by serum or small G proteins. In this study, we observed that NHE1 protein levels were very low in CHP1-deficient cells. As a result of the very low activity of NHE1 in these cells, we had considerable difficulty in performing the same type of experiments as those performed by Lin and Barber (19), such as serum activation. However, our results indicate that the primary role of CHP1 is stabilization of the NHE1 protein rather than regulation of NHE1 activity.

Expression of forms of CHP1 incapable of Ca\(^{2+}\) binding or myristoylation affect NHE1 protein levels (Fig. 6). However, the rescued NHE1 activities were lower than those observed in cells expressing wildtype CHPI. These results suggest that stabilization of NHE1 mediated by CHPI requires Ca\(^{2+}\) binding or myristoylation, but these factors may not be essential. Pang et al. (33) found that cells expressing CHPI-green fluorescent protein or mutant derivatives (E134A, E175A, E134H11022/E175A) exhibited similarly high Na\(^+/H^+\) exchange activity at an acidic pH, a result consistent with our observations. In our study, Na\(^+/\)H\(^+\) uptake activity by one NHE1 molecule in CHPI-deficient cells was \(-20\%\) of that of wildtype cells. It should be noted that this decreased activity was observed with endogenous NHE1 or engineered NHE1 lacking CHPI-binding capability, as previously described (34). Taken together, these results indicate that CHPI enhances the acidification-induced activity of NHE1 and that Ca\(^{2+}\) binding or myristoylation is not necessarily essential for NHE1 activities in intact cells.

Although we used chicken lymphoma DT40 cells, our study is likely applicable to mammalian systems. Chicken CHPI differs from mouse CHPI by only four amino acids, and functional motifs, such as EF-hands, are conserved in both. The hydrophilic region of chicken NHE1 also exhibits >85% homology to mouse NHE1. Moreover, the CHPI-binding site in this region of NHE1 is completely conserved among avian and mammalian species. Because chicken NHE1 on the cell surface showed two forms, slight differences such as glycosylation may exist. However, the results of the knockdown experiments in HeLa cells strongly support the notion that NHE1 stabilization by CHPI is not specific to chicken DT40 cells but rather is applicable to other types of cells and species.

Since CHPI has several binding partners, as described previously (1, 18, 20, 21, 24), loss of CHPI might affect several activities. However, we did not observe extensive decreases in cell growth or morphological changes in CHPI knockout cells nor did we find that the CHIP2 isoform was induced. In summary, in CHPI-deficient cells, extensive loss of NHE1 protein was observed. This result suggests that CHPI may be involved in the biogenesis or folding and/or stabilization of NHE1. On the basis of newly discovered physiological functions of CHPI, we conclude that CHPI primarily plays an essential role in stabilization of NHE1 at an early stage of biogenesis.

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