Dual functional significance of calcineurin homologous protein 1 binding to Na\(^{+}/\)H\(^{+}\) exchanger isoform 1

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Matsushita M, Tanaka H, Mitsui K, Kanazawa H. Dual functional significance of calcineurin homologous protein 1 binding to Na\(^{+}/\)H\(^{+}\) exchanger isoform 1. Am J Physiol Cell Physiol 301: C280–C288, 2011. First published May 4, 2011; doi:10.1152/ajpcell.00404.2010.—Calcineurin homologous protein 1 (CHP1) binds to the hydrophilic tail of the Na\(^{+}/\)H\(^{+}\) exchanger isoform 1 (NHE1). Previous gene knockout of CHP1 revealed that the loss of CHP1 caused a decrease in the total amount of NHE1, suggesting the destabilization of NHE1 molecules without CHP1 (Matsushita et al., Am. J Physiol Cell Physiol 293: C246–C254, 2007). However, Pang et al. (J Biol Chem 276: 17367–17372, 2001) reported that NHE1 without a CHP1 binding site was found in the plasma membrane, suggesting no requirement of CHP1 binding for plasma membrane localization of NHE1. Here, the functional significance of CHP1 binding to NHE1 was examined to resolve these contradictory results. In CV1 cells, which overexpressed wild-type NHE1, overexpression of CHP1 caused an increase in both the total amount of NHE1 and the colocalization of NHE1 and CHP1 at the plasma membrane. This provided new visual evidence of the localization of NHE1 from endoplasmic reticulum to the plasma membrane upon CHP1 binding. An immunoprecipitation assay showed that the expression of CHP1 reduced the ubiquitination of NHE1 and/or its associated proteins. Mutant NHE1s without CHP1 binding site exhibited a modest localization to the plasma membrane. After reaching the plasma membrane, these mutant NHE1s exhibited shorter half-lives than the wild-type NHE1 with CHP1. The results suggest a dual functional significance of CHP1 and its binding region: 1) binding of CHP1 stabilizes NHE1 and increases its plasma membrane localization by masking a NHE1 disposal signal, and 2) CHP1 binding is required for the antipporter activity.

Na\(^{+}/\)H\(^{+}\) exchanger; calcineurin homologous protein; ion transport; membrane protein; antipporter

MAINTENANCE of intracellular ionic environment is necessary for homeostasis in living cells. Regulation of ion concentrations in the cytoplasm is achieved by the balanced activities of ion pumps and transporters that are localized to the plasma membranes. Integration into the endoplasmic reticulum (ER), maturation at the ER and Golgi complex, translocation to plasma membranes, and disposal must be regulated for proper membrane transport protein function (26, 30). Recent studies revealed that the maturation and localization of membrane proteins requires various other binding partners (2, 8). However, their contributions to the processes are not fully understood.

Na\(^{+}/\)H\(^{+}\) exchangers (NHE) are secondary ion transporters found in cells from bacteria to mammals (19). These are involved in maintaining the concentration of H\(^{+}\), Na\(^{+}\), and/or K\(^{+}\) within cells or organelles by mediating the exchange of H\(^{+}\) for Na\(^{+}\) or K\(^{+}\). In mammals, Na\(^{+}/\)H\(^{+}\) exchangers are divided into two classes because of their intracellular localization (21). NHE1–5 (SLC9A1–5) are mainly localized in plasma membranes and play a key role in maintaining the intracellular concentration of Na\(^{+}\) and H\(^{+}\). NHE1 is a ubiquitous isoform and NHE2–5 are expressed in tissue-specific manners. NHE6–9 (SLC9A6–9) are localized to specific organelle membranes and are thought to be involved in the regulation of organelle pH (14, 18). Other SLC9 family members (SLC9B1–2 and SLC9C1–2) have also been identified, but their secondary structures differ from those of NHE1–9 (33) (http://www.bioparadigms.org/slc/).

NHE1 has a characteristic two-domain structure composed of an amino-terminal hydrophobic multispanning transmembrane domain (~500 amino acids) and a carboxyl-terminal hydrophilic domain (~300 amino acids) (21, 25). The hydrophilic domain faces the cytoplasm and various binding proteins are associated with this region (25). Many of these binding proteins mediate signals from extracellular stimuli and play important roles in regulating ion transport (7, 25, 28, 31, 32). The 22-kDa calcineurin homologous protein 1 (CHP1) binds to the juxtamembrane region in the cytoplasmic domain of plasma membrane NHEs (NHE1–5) (12, 22). Originally, Lin and Barber (12) reported that overexpression of CHP1 in CCL39 cells caused the inhibition of NHE1 activation induced by serum or small G proteins. Pang et al. (22) demonstrated that PS120 cells expressing NHE1 without CHP1 binding sites exhibited proper plasma membrane localization but lacked NHE1 activity. They concluded that CHP1 is an essential cofactor for the exchange activity of NHE1 but that it is not required for proper localization to plasma membranes. However, in CHP1 knockout chicken lymphoma cells (DT40), NHE1 protein levels were unexpectedly reduced to <10%, and very little NHE1 was localized to the plasma membrane (16). In CHP1 knockout cells, Na\(^{+}\) uptake activity by one NHE1 molecule was ~20% of wild-type cells. These data suggest that CHP1 plays an essential role in NHE1 activity as well as in the stabilization of NHE1 and the localization of NHE1 to the plasma membrane. However, this conflicts with the results of Pang et al. (22) in terms of the requirement for stability and localization of NHE1. The former study analyzed the mutant NHE1 lacking CHP1 binding sites, but the latter study analyzed wild-type NHE1 in the absence of CHP1. Thus the discrepancy may be due to unclarified functional significance regarding CHP1 binding and/or the CHP1 binding site. To address this issue, monkey cell lines (CV1) expressing wild-type NHE1 both with and without CHP1 or mutant NHE1 were established. The plasma membrane localization and the stability of NHE1 after localization to the plasma membrane were examined. The results suggested that the CHP1 binding site is involved in NHE1 degradation and masked by CHP1. Thus...
CHP1 binding has a dual functional significance, as it is important for the antiporter activity as well as the stabilization of NHE1 because of its ability to mask a degradation signal.

MATERIALS AND METHODS

Plasmid construction. For pcDNA5FRT-NHE1-HA, NHE1 cDNA (10) was attached with a NotI site-containing linker (GGC ATG GCG GCC GCC) and hemagglutinin (HA) tag (YPYDVPDYA) in the COOH-terminal end, amplified with PCR, and integrated into pcDNA5FRT (Invitrogen). The expression vectors encoding NHE1-HA with the modified CHP1-binding region was constructed with site-specific mutagenesis using overlapping reverse-complement primers (29). The primers for NHE1-HA[6Q] and NHE1-HA[ΔCBR] are GAA ACA ACG GCC TCC CAG AAC GAG GAG CAG ACA CAG CAG CTG GAC CAG CAC CAG ACA GCC CAG GAC GAC ATC TGT GG and GCA GAA AAC AAA GAG ATC TGA GGA CAT CTG TGG TCA TTA TG, respectively. For CHP1 expression vector, the cDNA of CHP1 was excised from pEF-BOS-CHP1 (15) with EcoRI and XhoI and integrated into pcDNA 3.1 (+) vector (Invitrogen). For the construction of the FLAG-tagged ubiquitin expression vector, FLAG-tagged ubiquitin was amplified from a human skeletal muscle cDNA library (Clontech) using PCR with the primers (forward primer containing the EcoRI site and FLAG-tag sequence: TGG AAT TCC ACC ATG GAC TAT AAA GAC GAC GGA CAT CTG TGG TCA TTA TG, and reverse primer containing the XhoI site: CTT CTA GAA CCA CCT CTC AGA CG), and then the amplified fragment was digested and integrated into pcDNA 3.1(+)-vector.

Cell culture and stable cell lines. To establish stable cell lines, we used a method that incorporates Flp recombinase-mediated site-specific integration (20). CV1-FRT cells, which have a single Flp recombination target (FRT) site within the genomic DNA, were obtained from Invitrogen. In this cell line, any gene of interest cloned into the Flp-targeted site in the circular expression plasmid can be specifically integrated into the single FRT site in the genomic DNA by a cotransfected FLP recombinase. The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37°C. To establish the cells expressing NHE1-HA (wild type, 6Q, and ΔCBR), cells at 10% confluence in 6-cm dishes were transfected with 0.25 µg of plasmid encoding NHE1-HA and 2.25 µg of plasmid encoding FLP recombinase (pOG44, Invitrogen) using 7.5 µl of Fugene6 (Roche Diagnostics). After 24 h of transfection, the medium was changed. After another 24 h, the cells were trypsinized and replated at 5% confluence in 10-cm dishes. The cells were incubated in medium containing 100 µg/ml hygromycin B for 2 wk, and drug resistant cells were isolated. To establish cells expressing CHP1, the cells were transfected with 2 µg of linearized CHP1 expression plasmid using 6 µl of Fugene6. The cells were incubated in the medium with 550 µg/ml G418 for about 2 wk and drug-resistant cells were isolated. The expression of NHE1-HA and CHP1 in isolated cells was confirmed with immunoblotting.

Immunoblotting. The sample preparation was performed without heat denaturing to minimize aggregation of NHE1, as described previously (5, 16). SDS-PAGE and immunodetection was performed as described previously (16). Mouse monoclonal (clone 16B12) and rabbit polyclonal anti HA-tag antibody were purchased from Covance (Princeton, NJ) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-FLAG M2 antibody conjugated with horseradish peroxidase was obtained from Sigma-Aldrich (St. Louis, MO). Anti-CHP1 antibody was prepared as described previously (15). Mouse monoclonal anti-PDI (Protein Disulfide Isomerase) antibody was purchased from Affinity BioReagents (Golden, CO).

Detection of ubiquitination. Cells in 3.5-cm dishes were transfected with the expression vector for FLAG-tagged ubiquitin. After 24 h, the cells were washed and lysed in phosphate-buffered saline (PBS) containing 1% Nonidet P-40, 1% Triton-X, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, and 1 µg/ml leupeptin with sonication for 90 s. The lysate was incubated for 20 min on ice and then debris was removed by centrifugation at 15,000 g. The supernatant was mixed with anti-HA antibody and Protein G-PLUS Agarose (Santa Cruz Biotechnology) and incubated for 3 h. The agarose beads were washed three times with PBS and mixed with the SDS-PAGE loading buffer.

Microscopy. Cells were fixed and stained with antibodies as described previously (16). For secondary antibodies, anti-rabbit IgG conjugated with Alexa Fluor 488 and anti-mouse IgG conjugated with Alexa Fluor 546 were obtained from Invitrogen.

Surface labeling assay. The cells were washed with PBS, incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) on ice for 30 min, and washed with PBS containing 0.1 M glycine (pH 7.4). For the pulse-chase experiment, the cells were incubated for the indicated times in normal medium at 37°C. Then the labeled cells were collected by scraping and centrifugation and lysed with PBS containing 1% Nonidet P-40, 1% Triton-X, 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) at 4°C for 120 min. The beads were washed twice with PBS and mixed with SDS-PAGE loading buffer containing 50 mM dithiothreitol.

RESULTS

Expression of CHP1 enhances the expression of NHE1 protein and its translocation to the plasma membrane. Previously, knockout of CHP1 in a chicken B lymphoma DT40 cell line reduced NHE1 expression (16); however, the role of CHP1 in the localization of NHE1 to the plasma membrane remains controversial and further studies are required to clarify this. CHP1 expression is difficult to visualize microscopically because of the small size of the DT40 cells and the small amount of NHE1 in CHP1-deficient or CHP1-knockdown cells (16). Thus, to study the localization of NHE1, a monkey cell line (CV1) that overexpressed NHE1 was established, and the effect of the overexpression of CHP1 on the localization and stabilization of NHE1 was examined. We also tried to knock down CHP1 using RNA interference, but the knockdown efficiency in CV1 cells was not satisfactory. Moreover, CV1 cells expressing mutant NHE1 that lacked CHP1 binding sites were created. In this type of experiment, the variety of expression levels in the wild-type or mutant NHE1, where each had different transfection efficiencies, and the random integration of the gene in stably expressing cells made the interpretation of the results difficult. Therefore, the FLP-FRT system was used for the site-specific integration of NHE1. This ensured that the copy number of the integrated NHE1 gene in CV1 cells was one (20). An NHE1-HA expressing CV1 cell line (NHE1-HA cell) was established (Fig. 1A). In this cell line, the expression level of HA-tagged NHE1 was ~30-fold higher than that of endogenous NHE1 in the parental cell line (data not shown). Next, a CHP1 expression vector was introduced into NHE1-HA cells (NHE1-HA/CHP cell) (Fig. 1). In NHE1-HA/CHP1 cells, CHP1 expression was 20-fold higher than that in NHE1-HA cells (Fig. 2, A and B), and the expression level of NHE1 was 10-fold higher than that in NHE1-HA cells (Fig. 1, A and B). Previously, the level of ubiquitinated NHE1 was higher in CHP1 knockout DT40 cells, which suggested that ubiquitin-dependent degradation was involved in the reduced expression of NHE1 (16). Therefore, the ubiquitination of NHE1 in NHE1-HA and NHE1-HA/CHP1 cells was analyzed. To do
this, the cells were transfected with FLAG-tagged ubiquitin, and HA-tagged NHE1 was immunoprecipitated using HA antibody. As a result, ubiquitinated-NHE1, or ubiquitinated NHE-associated proteins, probed with FLAG antibody in NHE1-HA cells was detected at a higher level (Fig. 3, right) than that found in NHE1-HA/CHP1 cells, although the expression level (Fig. 1) and immunoprecipitated amount of NHE1-HA protein (Fig. 3, left) in NHE1-HA cells was lower than that in NHE1-HA/CHP1 cells. These results showed that the overexpression of CHP1 stabilized NHE1 and were in accordance with earlier results from this laboratory. Thus this system is suitable for analyzing the intracellular localization of NHE1 when CHP1 is limiting.

Immunoblot analysis (Fig. 1) showed several NHE1 bands, indicating that different glycosylation and/or oligomeric forms had been separated during electrophoresis (6). In NHE1-HA/CHP1 cells, the highly glycosylated form (see ** in Fig. 1) was more evident than the core glycosylated form (see * in Fig. 1).

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**Fig. 1.** Expression of hemagglutinin-tagged (HA) Na+/H+ exchange (NHE-HA) protein in stable cell lines. The CV-1FRT cell line integrated with the NHE1-HA expression cassette was established (NHE1-HA cell line). Then NHE1-HA cells were transfected with a calcineurin homologous protein 1 (CHP1) expression vector, and a stable cell line expressing additional CHP1 was established (NHE1-HA/CHP1 cell line). *A*: expression of NHE1-HA proteins was analyzed by immunoblotting with anti-HA antibody. The sample from NHE1-HA cells (relative protein amount is 1) and a series of serially diluted samples (1 to 1/100) from NHE1-HA/CHP1 cells were applied. *Core glycosylated form.* **Highly glycosylated form.***Oligomeric form.* B: relative amount of expressed NHE1-HA protein in the stable cell lines. The expression level of NHE1-HA protein was quantified with ImageJ software and results are presented as means ± SD (n = 3).

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**Fig. 2.** Expression level of CHP1 protein in stable cell lines. *A*: expression of CHP1 proteins was analyzed by immunoblotting with anti-CHP1 antibody. The sample from NHE1-HA cells (relative protein amount is 1) and a series of serially diluted samples (1 to 1/200) from NHE1-HA/CHP1 cells were applied. *Relative amount of CHP1 protein in the stable cell lines.* Data are presented as means ± SD (n = 3).
The slower migration of the highly glycosylated form of NHE1 was attributed to O-linked glycosylation (6), which suggested that the maturation and/or translocation of NHE1 from ER to the plasma membrane was promoted by CHP1 in NHE1-HA/CHP1 cells.

Next, the intracellular localization of NHE1-HA and CHP1 was examined. In NHE1-HA cells, NHE1 and CHP1 were both localized to intracellular compartments (Fig. 4A). The fluorescent signal of NHE1-HA was localized along the pattern of tubular network of ER, which was visualized with an ER marker (the protein disulfide isomerase) (Fig. 4B). In NHE1-HA/CHP1 cells, NHE1 and CHP1 were not only detected in intracellular compartments but also in the periphery (Fig. 4C), where they appeared to colocalize at the cell membrane (Fig. 4C, arrowheads). NHE1 staining was also distributed uniformly throughout the cell. Such uniform staining of membrane proteins implies plasma membrane localization and suggests that the transport of NHE1 from the ER to the plasma membrane was enhanced by the addition of CHP1. Next, we examined the expression of NHE1 at the plasma membrane using a surface biotinylation assay (Fig. 5). The surface expression of NHE1 in NHE1-HA/CHP1 cells was 30-fold higher than that in NHE1-HA cells (Fig. 5B). As described above, the total expression level of NHE1 in NHE1-HA/CHP1 cells was 10-fold higher than that in NHE1-HA cells. This indicated that CHP1 is involved not only in the stabilization of NHE1 but also in its localization to plasma membranes.

Expression and localization of NHE1 lacking the CHP1-binding site. Previous studies identified amino acid residues in NHE1 that are involved in CHP1 binding (22). Cell lines that express NHE1 lacking these amino acid residues were established (Fig. 6). NHE1-HA[6Q] cells expressed HA-tagged NHE1 with six amino acid substitutions in the CHP1 binding site (6Q, Fig. 6, A and B). NHE1-HA[ΔCBR] cells expressed a mutant NHE1 with a deletion in the CHP1-binding region (CBR, a.a. 522–538) (ΔCBR, Fig. 6, A and B). As a result, the mutant NHE1 exhibited a localization pattern that was similar to that of NHE1-HA/CHP1 cells (Fig. 6C). Unlike the results with NHE1-HA/CHP1 cells (Fig. 4C), the colocalization of mutant NHE1 and CHP1 in the cell periphery was not observed, which confirmed the loss of the CHP1 binding site in NHE1. The amount of total and surface NHE1 was quantified using immunoblot analysis and surface labeling. The total amount of mutant NHE1-HA in NHE1-HA[ΔCBR] cells was severalfold higher than the total amount of the wild-type NHE1-HA in NHE1-HA cells (Fig. 7A). The surface amount of the mutant protein was also higher than that of the wild-type protein (Fig. 7B). These results indicated that wild-type NHE1-HA and mutant NHE1 lacking CHP1-binding sites are not processed through stabilization or disposal steps in the same manner.

Stabilization of NHE1 in the plasma membrane by CHP1. The amount of NHE1 protein in the plasma membrane was reduced in NHE1-HA cells without CHP1 overexpression (Fig. 5) and in the CHP1-knockout DT40 cells (16) compared with the NHE1-HA/CHP1 cells that had sufficient amounts of CHP1. However, surface expression of mutant NHE1 lacking the CHP1-binding site was observed (Figs. 6 and 7). This suggested that mutant NHE1 passed through the stabilization and disposal steps in early stages of biogenesis and that it finally localized to the plasma membrane (16). To analyze the stability of NHE1 upon reaching the plasma membrane, a pulse-chase experiment using pulse-surface labeling was performed. After a 9-h chase period, ~70% of NHE1-HA protein remained in NHE1-HA/CHP1 cells (Fig. 8). In NHE1-HA[ΔCBR] cells, ~20% of mutant NHE1-HA protein remained after a 9-h chase (Fig. 8). NHE1-HA protein in NHE1-HA cells exhibited a decrease of the remaining amount in a similar manner to mutant NHE1-HA proteins. These results suggested that mutant NHE1 (6Q and [ΔCBR]) were not as stable as the wild-type NHE1-HA in NHE1-HA/CHP1 cells, even if the mutant NHE1 became localized to the plasma membrane. After biotin label and chase, the NHE1 protein in NHE1-HA/CHP1 cells (Fig. 8A), the positions are indicated by #). The conformation of NHE1-HA proteins without binding to CHP1, NHE1-HA[6Q], and NHE1-HA[ΔCBR] may be different from that of NHE1-HA with binding to CHP1. Taken together, these results suggested that NHE1 was stabilized by CHP1 even after localization to the plasma membrane.

DISCUSSION

It was previously found that the amount of NHE1 protein in CHP1-knockout DT40 cells was reduced to <10% of control and that it did not localize to the plasma membrane. In addition, when CHP1 was expressed in the CHP1-knockout cells, protein expression of NHE1 was recovered and NHE1 was translocated to the plasma membrane (16). These results conflicted with those of Pang et al. (22), who reported that CHP1 was not required for the translocation of NHE1 to
plasma membranes. To solve this discrepancy, experiments using the CV1 cell line where wild-type and mutant NHE1 could be analyzed were performed. This cell line also provided a way to visually examine the effects of CHP1 binding on the localization of NHE1. In addition, the stability of NHE1 after reaching the plasma membrane was examined, which had not been done previously.

The enhancement of localization to plasma membrane in NHE1-HA/CHP1 cells compared with NHE1-HA cells is well consistent with our previous observation in CHP1-knockout cells (16). Although these results imply the requirement of CHP1 for the localization of NHE1 to the plasma membrane, the mutant NHE1s in NHE1-HA[6Q] and NHE1-HA[ΔCBR] cells exhibited higher total and surface expression than NHE1 in NHE1-HA cells, which is essentially the same result as that found by Pang et al. using PS120 cells (22). The mutant NHE1 might form more stable conformations than the wild-type NHE1 without CHP1. An alternative is that the CHP1 binding region of NHE1 acts as a target for the machinery for the retention, retrieval, and/or disposal of NHE1. Although CHP1 binds to this region and stabilizes the conformation of NHE1, CHP1 may also mask the putative retention, retrieval, and/or disposal target sequence, leading to the transport of NHE1 to the plasma membrane. Wild-type NHE1 without binding of CHP1 may expose the target sequence and eventually be recognized by a putative disposal system. When the target sequence is altered, the mutant NHE1s might escape from recognition by retention, retrieval, and/or disposal systems. Possible candidate for the disposal system is a ubiquitin-dependent machinery. In concordance, NHE1-HA and/or its binding proteins in the absence of CHP1 was found to be more highly ubiquitinated than NHE1-HA in the presence of CHP1. If NHE1 is directly ubiquitinated (see discussions in the next paragraph), the CHP1 binding site may be recognized by a ubiquitin ligase. Two lysine-rich sequences (KKKQETK and KKYVKK) that could possibly be ubiquitinated reside close to the CHP1-binding region (aa. 522–538). An experiment using recombinant proteins comprising a CHP1-

![Fig. 4. Intracellular localization of NHE1-HA and CHP1. NHE1-HA (A and B) and NHE1-HA/CHP1 (C) cells were fixed and stained with anti-HA and anti-CHP1 (A and C) or anti-PDI (B) antibody. The second row in the B shows magnified pictures of the regions indicated by white rectangles in the first row in the B. Colocalization of NHE-HA and CHP1 in the cell periphery is indicated by the arrowheads. Bar, 25 μm.](http://ajpcell.physiology.org/content/301/4/C284)
binding region plus the two lysine-rich sequences suggested that S59K\(\text{KKYVKK}^{564}\) is associated with ubiquitination (see Supplemental Fig. S1 at the AJP-Cell Physiology website). The two lysine-rich sequences are also thought to be involved in binding phosphatidylinositol 4,5-bisphosphate (1). A recent study showed that NHE1 was ubiquitinated on a.a. 1–675 but not on a.a. 1–550 (27). Since many lysine residues were found in a.a. 550–675 in addition to the second lysine-rich cluster, it

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**Fig. 5. Surface expression of NHE1-HA.**

A: NHE1-HA proteins on the cell surface were labeled with aminoreactive biotin derivatives and analyzed by immunoblotting with anti-HA antibody (see MATERIALS AND METHODS). The sample from NHE1-HA cells (relative protein amount is 1) and a series of serially diluted samples (1/5 to 1/200) from NHE1-HA/CHP1 cells were applied. B: relative amount of surface NHE1-HA protein quantified with immunoblotting. Data are presented as means ± SD.

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**Fig. 6. Intracellular localization of mutant NHE1-HA protein deficient in CHP1 binding.**

A: sequences of membrane-proximal region in wild-type (WT) and mutant NHE1. The substituted residues in NHE1-HA[6Q] were underlined. B: expression of WT and mutant NHE1-HA proteins was analyzed by immunoblotting with anti-HA antibody. *Core glycosylated form. **Highly glycosylated form. ***Oligomeric form. C: intracellular localization of WT or mutant NHE1-HA proteins and CHP1. The cells were fixed and stained with anti-HA and anti-CHP1 antibody. Bar, 10 μm.
was unclear which ubiquitinated site is involved in CHP1-dependent stabilization of NHE1.

Previous studies report CHP1 localization not only in the cytosol and at the plasma membrane but also at the ER membrane and in microtubule networks, and that CHP1 interacts with many binding partners involved in intracellular transport (3, 4, 17). Overexpression of CHP1 may facilitate stabilization and localization by affecting vesicular trafficking and

Fig. 7. Expression level of mutant NHE1-HA protein deficient in CHP1 binding. A: relative amount of expressed NHE1-HA protein in the stable cell lines. The expression level of NHE1-HA protein in a total cell lysate was quantified with ImageJ software, and results are presented as means ± SD (n = 3). *P < 0.05 by t-test versus NHE1-HA. #Amount of NHE1-HA[ΔCBR] were larger than that of NHE1-HA in the all trials. B: relative amount of surface-labeled NHE1-HA protein quantified with immunoblotting (see MATERIALS AND METHODS). Data are presented as means ± SD (n = 3).

Fig. 8. Stability of wild-type and mutant NHE1-HA proteins. The cells were labeled with amine-reactive biotin-derivative for 30 min and chased for the indicated times. Then biotin-labeled NHE1-HA proteins were isolated and detected (see MATERIALS AND METHODS). A: relative amount of NHE1-HA proteins. Data represent the total intensity of each lane in the immunoblot using NHE1-HA antibody (shown in B). The values and bars are the average of two independent experiments and the differences, respectively. B: immunoblotting of biotin-labeled NHE1-HA protein remaining after indicated times. The labeled NHE1-HA proteins were isolated with NeutrAvidin beads and detected with anti-HA antibody. **Highly glycosylated form. #Slower migrating species (see RESULTS for details).
microtubule-dependent transport without any direct interaction with NHE1. However, in cells expressing mutant NHE1, which lacks the CHP1 binding region, overexpression of CHP1 did not affect the amount or the localization of mutant NHE1 (Supplemental Fig. S2). Thus it is suggested that the increased stability and translocation of NHE1 is mediated by the direct binding of CHP1. On the other hand, NHE1 has many different binding partners. The results of the ubiquitination assay (Fig. 3) do not completely exclude the possibility that the other coimmunoprecipitated binding partners were also ubiquitinated. However, considering that a recent study showed that NHE1 was directly ubiquitinated (27), we supposed that this was also the case in our experiment. We also observed that CHP1 had a stabilizing effect upon NHE1 at the plasma membrane (Fig. 8). Could the increase in NHE1 expression at the plasma membrane be explained only by this stabilization effect? The half-life of NHE1 in CHP1-expressing cells (NHE1-HA/CHP1 cells) was approximately three to fourfold longer than that in NHE1-HA cells (Fig. 8). In general, the amount of protein expressed at the cell surface during the steady state is proportional to its half-life. Thus only three- to fourfold higher levels of expression at the plasma membrane could be explained by the three- to fourfold longer half-life observed in this study. However, the amount of surface NHE1 expressed by NHE1-HA/CHP1 cells was ~30-fold higher than that in cells expressing NHE1 alone (Fig. 5). Such a large difference would require other effects, such as enhanced transport from the ER. Thus the increased expression of NHE1 at the plasma membrane was not only due to a longer half-life but also to increased stabilization before reaching the cell surface.

Although the mutant NHE1s defective in CHP1-binding could reach the plasma membrane, these mutant NHE1s had a short half-life in the plasma membrane (Fig. 8). This suggested that NHE1 without binding to CHP1 proteins may have an unstable conformation even if they can localize to the plasma membrane. Consistent with this, previous studies showed that cells expressing mutant NHE1 with deletions or substitutions in the CHP1 binding region were defective in the Na+/H+ antiport activity (22).

In previous studies using the PS120 cell line with defective NHE1 (22), the NHE1-GFP fusion overexpressed using an expression vector was localized at plasma membranes even without overexpression of CHP1. These observations are not consistent with the present results (Fig. 4). Endogenous NHE1 is not expressed in PS120 cells (9, 11, 24). The abundant, free CHP1 in the PS120 cells may have bound toand stabilized the highly unstable conformation even if they can localize to the plasma membrane. Consistent with this, previous studies showed that NHE1 without binding to CHP1 proteins may have an unstable conformation even if they can localize to the plasma membrane. Consistent with this, previous studies showed that NHE1 without binding to CHP1 proteins may have an unstable conformation even if they can localize to the plasma membrane.

It has been reported that CHP1 is required for NHE1 antiport activity (16, 22). The results of the present study imply a new functional significance of CHP1 for stabilization of NHE1. Thus the binding of CHP1 to NHE1 has dual functions. First, the binding of CHP1 stabilizes NHE1 and increases its plasma membrane localization by masking a NHE1 disposal signal, and second, CHP1 binding is required for the antipporter activity of NHE1.

CHP1 is expressed ubiquitously and has a closely related tissue- or cell-specific isoform. CHP2 is similar in size and shares 60% amino acid sequence identity with CHP1. The enhanced plasma membrane localization of NHE1 was observed with fluorescence microscopy when CHP2 was expressed in NHE1-HEA cells (data not shown). Another tissue-specific CHP-related protein tescalcin (13, 23) has been identified. Unlike CHP2, tescalcin is different in size and shares <30% amino acid sequence identity with CHP1. A recent study reported that tescalcin (CHP3) is involved in the maturation, cell surface stability, and optimal transport of NHE1 (34). Thus CHP proteins may share similar roles in the stabilization of NHE1.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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