N-Myristoylation and Ca\textsuperscript{2+} Binding of Calcineurin B Homologous Protein CHP3 Are Required to Enhance Na\textsuperscript{+}/H\textsuperscript{+} Exchanger NHE1 Half-life and Activity at the Plasma Membrane\textsuperscript{[S]}

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**Background:** CHP3 is an N-myristoylated Ca\textsuperscript{2+}-binding protein that up-regulates the cell surface expression and stability of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger NHE1 isoform.

**Results:** N-Myristoylation or the Ca\textsuperscript{2+}-binding site of CHP3 regulates the half-life and activity of NHE1 at the cell surface.

**Conclusion:** CHP3 possesses a Ca\textsuperscript{2+}-myristoyl switch mechanism to promote optimal NHE1 activity at the cell surface.

**Significance:** These findings provide fundamental insight into the molecular mechanisms that regulate NHE1.

Calcineurin B homologous proteins (CHP) are N-myristoylated, EF-hand Ca\textsuperscript{2+}-binding proteins that regulate multiple cellular processes, including intracellular pH homeostasis. Previous work has shown that the heart-enriched isoform, CHP3, regulates the plasmalemmal Na\textsuperscript{+}/H\textsuperscript{+} exchanger NHE1 isoform by enhancing its rate of oligosaccharide maturation and exocytosis as well as its half-life and transport activity at the cell surface (Zaun, H. C., Shrier, A., and Orlowski, J. (2008) *J. Biol. Chem.* 283, 12456–12467). However, the molecular basis for this effect is not well understood. In this report, we investigated whether the N-myristoylation and Ca\textsuperscript{2+}-binding domains of CHP3 are important elements for regulating NHE1. Mutation of residues essential for either N-myristoylation (G2A) or calcium binding (D123A) did not prevent the interaction of CHP3 with NHE1, although the D123A mutant no longer showed elevated binding to NHE1 in the presence of Ca\textsuperscript{2+} when assessed using *in vitro* binding assays. Disruption of either site also did not impair the ability of CHP3 to stimulate the biosynthetic processing and trafficking of NHE1 to the plasma membrane or did it affect the H\textsuperscript{+} sensitivity of the exchanger. However, they did significantly reduce the cell surface half-life and near maximal transport velocity of NHE1 to a similar extent. Simultaneous mutation of both sites (G2A/D123A) gave results identical to the individual substitutions. This finding suggests that both domains in CHP3 are interdependent and may function cooperatively as a Ca\textsuperscript{2+}-myristoyl switch mechanism to selectively stabilize the NHE1-CHP3 complex at the cell surface in a conformation that promotes optimal transport activity.

Regulation of intracellular pH (pH\textsubscript{i}) is a fundamental physiological process of all living cells. In mammals, precise control of pH\textsubscript{i} involves the coordinated activities of several distinct solute carriers that conduct the transmembrane fluxes of H\textsuperscript{+} or HCO\textsubscript{3}\textsuperscript{-}, usually directly coupled to the movement of another ion (1). Of these, one of the major mechanisms for protecting cells from excess intracellular acidification involves the coupled countertransport of alkali cations such as Na\textsuperscript{+}, but in some cases also K\textsuperscript{+}, for H\textsuperscript{+} across the cell surface and are simply referred to as Na\textsuperscript{+}/H\textsuperscript{+} exchangers or antiporters (NHE\textsuperscript{2}/NHX/NHA).

The NHE1 isoform has been studied extensively because it is present in most cells and makes vital contributions to not only cytoplasmic pH homeostasis but also an array of other physiological processes, such as cell volume regulation (2), shape (3), adhesion and spreading (4), migration (5), proliferation (2, 6), differentiation (7, 8), and apoptosis (9, 10). The central involvement of NHE1 in such diverse physiological phenomena has prompted searches for unique as well as common regulatory factors that might underlie these relationships. Not surprisingly, numerous hormones, growth factors, and second messengers such as Ca\textsuperscript{2+} have been found to regulate NHE1 activity by either phosphorylation-dependent or -independent mechanisms that, in several cases, involve the subsequent binding of various effector molecules to the cytoplasmic C terminus of the transporter (11–13). One such class of interacting proteins is a family of N-myristoylated, EF-hand Ca\textsuperscript{2+}-binding proteins called the calcineurin B homologous proteins (CHPs) (14).

The CHP proteins are of particular interest because they are critical for optimal basal as well as stimulus-mediated regulation of the plasmalemmal NHEs. They compose a family of three isoforms (CHP1–CHP3) that share homology with the calcineurin B subunit of the phosphatase, calcineurin, and indeed are capable of regulating the phosphatase activity of the calcineurin A catalytic subunit (15, 16). CHP1 (also known as p22) is widely expressed and sets the resting pH sensitivity of NHE1 in the physiological neutral range, but it also confers
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responsiveness to various signaling molecules (17, 18). By contrast, CHP2 expression is detected mainly in normal intestinal epithelia (19), but it is induced in several malignant cell types where it constitutively enhances the pH sensitivity of NHE1 in the absence of peripheral stimulatory signals, resulting in a more alkaline cytoplasm that promotes their survival (20–22). The third isofrom of the CHP family, CHP3 or tescalcin, was originally discovered as an autosomal gene whose mRNA transcript was detected in mouse developing testis (23). However, in adult animals, its expression is restricted mainly to heart, brain, stomach, and hematopoietic cells (16, 24). Functionally, CHP3 is distinguished by its ability to positively enhance multiple biochemical properties of NHE1. These include elevating its rate of post-translational maturation along the exocytic pathway, its half-life at the plasma membrane, and its maximal transport velocity without affecting its intracellular H⁺ affinity (25). A more recent study indicated that increased stabilization of the NHE1 protein may also be conferred by the CHP1 isoform (26).

How the CHP proteins are able to differentially modulate various facets of NHE1 function is poorly understood. Previous studies have shown that all three CHP proteins contain an N-myristoylated consensus site and at least one functional EF-hand Ca²⁺-binding domain (16, 17). N-Myristoylation is known to promote the reversible tethering of proteins to the inner leaflet of membrane bilayers, thereby providing an effective means of controlling the membrane targeting and function of certain soluble proteins (27, 28). By comparison, EF-hand Ca²⁺-binding proteins are known to undergo Ca²⁺-dependent conformational changes that modulate their function or influence the activity of their effectors (29). Interestingly, some proteins contain both elements that function cooperatively as a Ca²⁺-myristoyl switch to control specific calcium-sensitive membrane processes (30). However, in the case of the NHE1-CHP1 complex, the Ca²⁺-myristoyl switch mechanism does not appear operational as it binds Ca²⁺ constitutively to two of its four predicted EF-hand motifs (EF3 and EF4) due to its high nanomolar affinity for Ca²⁺ (apparent \( K_d \approx 2 \text{nM} \)) under resting physiological conditions (17, 18). Mutation of its myristoylation site did not affect the membrane trafficking or activity of NHE1, whereas disruption of either Ca²⁺-binding domain significantly reduced the H⁺ affinity and activity of the exchanger as well as its responsiveness to various stimuli (17). Hence, it was proposed that the Ca²⁺-binding domains in CHP1 might serve a more structural rather than Ca²⁺-sensing/ regulatory role. By comparison, CHP3 contains only a single functioning EF-hand Ca²⁺-binding domain (EF3) that binds Ca²⁺ with lower micromolar affinity and therefore may behave differently than CHP1 (16).

In this study, we investigated this possibility and found that N-myristoylation and Ca²⁺ binding of CHP3 are not required for the interaction between NHE1 and CHP3 nor are they necessary for CHP3 to enhance the biosynthetic maturation of NHE1. However, Ca²⁺ enhances CHP3 binding to NHE1, and loss of either N-myristoylation or Ca²⁺ binding similarly decreased the stability and transport activity of NHE1 at the cell surface. Simultaneous disruption of both sites gave results comparable with the individual mutations. This finding suggests that both domains in CHP3 are structurally linked and may operate as a Ca²⁺-myristoyl switch mechanism to selectively stabilize the NHE1-CHP3 complex at the cell surface in an arrangement that enables optimal exchange activity.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies to a decapetide derived from influenza virus hemagglutinin (HA) were purchased from Covance Inc. (Berkeley, CA) and to the peptide of the c-myc protooncogene (myc) from Millipore (Temecula, CA). Polyclonal antibodies to the HA-epitope and Myc epitope were purchased from Abcam Inc. (Cambridge, MA) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively, and antibodies specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Abcam. All Alexa Fluor®-conjugated goat antimouse or anti-rabbit IgG antibodies were purchased from Molecular Probes (Eugene, OR).

Vent polymerase, DNA ligase, restriction endonucleases, as well as protein and DNA markers were purchased from New England Biolabs (Ipswich, MA). α-Minimum essential medium, fetal bovine serum (FBS), penicillin/streptomycin, genetin (G418), trypsin-EDTA, and Lipofectamine-2000™ transfection reagent were all purchased from Invitrogen. Carrier-free 12NaCl (range of specific activity, 900–950 mCi/mg; concentration, ~10 mCi/ml) was obtained from PerkinElmer Life Sciences. Amiloride hydrochloride, nigericin, and ouabain were all purchased from Sigma, and complete protease inhibitor mixture tablets were obtained from Roche Diagnostics. All other chemicals and reagents were purchased from BioShop Canada (Burlington, Ontario, Canada), Sigma, or Fisher and were of highest grade available.

cDNA Construction and Mutagenesis—A mammalian expression vector under the control of the enhancer/promoter region from the immediate early gene of human cytomegalovirus (pCMV) and expressing either the cDNA of NHE1 containing a C-terminal hemagglutinin (HA) epitope tag (NHE1HA) or CHP3/tescalcin cDNA constructed to contain a Myc epitope at its C terminus (CHP3myc) were constructed as described previously (25).

Mutations that disrupt the N-myristoylation (G2A) and EF-hand Ca²⁺-binding sites (D123A) of CHP3myc as identified by Gutierrez-Ford et al. (16) were accomplished by PCR mutagenesis. All constructs were sequenced to confirm the presence of the desired mutations and to ensure that other random mutations were not introduced.

Cell Culture and Transfection—A cell line devoid of endogenous Na⁺/H⁺ exchanger activity derived from Chinese hamster ovary fibroblasts (CHO), termed AP-1 (31), was maintained in α-minimum essential medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 units/ml/100 μg/ml), and 25 mm sodium bicarbonate. Cells were incubated in a humidified atmosphere of 95% air, 5% CO₂ at 37 °C. For AP-1 cells expressing either NHE1HA or coexpressing NHE1HA along with either wild-type or mutated CHP3myc a total of 2 μg of DNA was transfected in a 6-well plate using Lipofectamine™-2000 reagent according to the manufacturer’s recommended procedure. Twenty four hours post-transfection, the cells were split into 10-cm dishes at a dilution of 1:10 and 1:50 and then selected for cells that stably express...
NHE1
\textsubscript{HA} by testing their ability to survive repeated challenges of intracellular acid loading over a 2-week period, as described previously (32). Cells stably expressing CHP3
\textsubscript{myc} were selected in α-minimum essential medium culture medium supplemented with the aminoglycoside antibiotic gentamicin (G418) (600 μg/ml) over a 2–4-week period.

**Coimmunoprecipitation and Western Blotting—**Coimmunoprecipitations of wild-type NHE1
\textsubscript{HA} and wild-type or mutant forms of CHP3
\textsubscript{myc} (G2A, D123A, and G2A/D123A) were performed in 10-cm dishes by transfecting AP-1 cells stably expressing NHE1
\textsubscript{HA} with 10 μg of the desired CHP3
\textsubscript{myc} constructs using Lipofectamine\textsuperscript{TM}-2000 according to the manufacturer's recommended procedure. Twenty four hours post-transfection, cell lysates were obtained by washing cells in ice-cold PBS and adding 1 ml of radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 2.5% deoxycholate, 0.5% Nonidet P-40, and protease inhibitors). For Ca\textsuperscript{2+} dependence of NHE1
\textsubscript{HA}/CHP3
\textsubscript{myc} coimmunoprecipitation, RIPA buffer was either supplemented with 1 mM Mg\textsuperscript{2+} and 2 mM EDTA or 0.1 mM Ca\textsuperscript{2+} without EDTA. Cell were scraped from the dish and incubated for 20 min at 4 °C, followed by centrifugation for 20 min at 4 °C to pellet cellular debris. Supernatants were then pre-cleared with 100 μl of a 50% protein G-Sepharose (GE Healthcare) slurry for 2 h at 4 °C. After brief centrifugation to remove the protein G-Sepharose and retaining a small fraction for Western blotting, the remaining supernatants were incubated with 5 μl of primary rabbit polyclonal antibody against either the HA or Myc epitope and incubated overnight at 4 °C with gentle rocking. Protein conjugates were then eluted by SDS sample buffer (2% SDS, 50 mM Tris acidified using the NH\textsubscript{4}Cl technique, and the initial rates of NHE1 activity was defined as the amiloride-inhibitable fraction of NHE1 activity was assessed using a radioisotope influx assay. Briefly, to measure NHE1 activity at near maximal velocity, cells were acidified using the NH\textsubscript{4}Cl technique, and the initial rates of Na\textsuperscript{+} influx were measured in the absence and presence of the NHE1 inhibitor amiloride (1 mM), as described previously (25). NHE1 activity was defined as the amiloride-inhibitable fraction of the total radioisotope influx. Protein content was determined using the Bio-Rad DC protein assay procedure.

To measure the NHE1 activity as a function of the expression of the different CHP3
\textsubscript{myc} constructs, 10-cm dishes of AP-1 cells stably expressing NHE1
\textsubscript{HA} were grown to subconfluence and transfected with an increasing ratio of CHP3
\textsubscript{myc}-containing plasmids relative to empty vector (0–10 μg) using Lipofectamine\textsuperscript{TM}-2000. Twenty four hours post-transfection, cells were split into 24-well plates (6-wells per transfection) for parallel Western blotting analyses; the cells were incubated for a further 24 h prior to the analyses. NHE1
\textsubscript{HA} activity was also measured as a function of the intracellular H\textsuperscript{+} concentration (pH\textsubscript{i}) by clamping pH\textsubscript{i} over the range of 5.4 to 7.4 using the K\textsuperscript{+}/H\textsuperscript{+} exchange ionophore nigericin as described previously (34).

**Cell Surface Biotinylation and Pulse-Chase Assay—**To determine the relative amount of cell surface NHE1
\textsubscript{HA}, as a function of the expression of CHP3
\textsubscript{myc} (wild-type, G2A, D123A, G2A/D123A), we used a cell surface biotinylation assay as described...
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previously (25). Briefly, AP-1 cells were grown to subconfluence on 10-cm dishes and transfected with 8 μg of expression vector containing NHE1HA along with an increasing ratio of the different CHP3myc cDNA constructs (0–2 μg) to empty expression vector using Lipofectamine™-2000. A green fluorescent protein (GFP) expression vector (1 μg) was also transfected as a control for transfection efficiency. Forty eight hours post-transfection, cells were placed on ice, and surface proteins were covalently modified with sulfo-NHS-SS-biotin (Thermo Scientific, Rockford, IL), a water-soluble, membrane-impermeable, thiol-cleavable, and amine-reactive biotinylation reagent. Following the addition of quenching buffer (20 mM glycine in PBS), cell lysates were obtained in RIPA buffer by scraping cells and incubating for 20 min on ice, followed by centrifugation for 20 min to remove cellular debris. A small fraction of supernatant was removed for Western blotting, and the remaining supernatant was incubated with a 50% NeutrAvidin®-agarose slurry (Thermo Scientific) in RIPA buffer overnight at 4 °C. The bound biotinylated protein complexes were isolated by centrifugation and then subjected to SDS-PAGE and immunoblot analyses.

The cell surface stability of NHE1HA in relation to the expression of CHP3myc wild-type or mutants was determined through a pulse-chase of biotinylated NHE1HA as described previously (25). Briefly, 6-well plates containing AP-1 cells expressing either NHE1HA alone or coexpressing NHE1HA along with CHP3myc wild-type or mutants forms (G2A, D123A, G2A/D123A) were grown to ~90% confluence, and cell surface proteins were biotinylated and quenched as described above. After extensive rapid washing to remove excess biotin, cells were returned to growth media supplemented with 10% FBS and cultured at 37 °C in 5% CO2, 95% air for various time points with fresh media added every 12 h to maintain cell viability. At the indicated time points, cells lysates and biotinylated proteins were obtained as described above and subjected to SDS-PAGE and immunoblotting.

The relative band intensities of the proteins for each time point on the Western blots were obtained through multiple exposures of the same blot to ensure the signal was within the linear range of the x-ray film. Densitometry measurements were obtained using ImageJ™ image processing software.

RESULTS

Role of N-Myristoylation and Ca2+ Binding in the Interaction between NHE1 and CHP3—To characterize the biological significance of N-myristoylation and Ca2+ binding of CHP3 in relation to its regulation of NHE1, the critical glycine residue of the N-myristoylation motif position 2 and the crucial aspartic acid in the EF-hand Ca2+ -binding motif at position 123 were mutated separately to alanine (G2A and D123A, respectively) (amino acid sequences shown in supplemental Fig. S1). Although the CHP proteins contain four potential EF-hand domains, only the third domain in CHP3 that contains Asp-123 was shown previously to bind Ca2+ (16).

In a prior study (25), we demonstrated that NHE1 interacts with CHP3 when coexpressed in intact cells. To assess whether this interaction is dependent on N-myristoylation or Ca2+ binding of CHP3, each of the CHP3 constructs (wild-type (WT), G2A, or D123A) tagged at their C terminus with a Myc epitope (CHP3myc) was transiently transfected in the Chinese hamster ovary AP-1 cell line that is devoid of endogenous NHE1 but stably expresses an HA epitope-tagged form of NHE1 (NHE1HA). At 24 h post-transfection, cell lysates were obtained and incubated with either a mouse polyclonal antibody recognizing the HA epitope of NHE1HA or an IgG control for transfection efficiency. Forty eight hours post-transfection, cell lysates were prepared, and NHE1HA-containing protein complexes were immunoprecipitated with a rabbit polyclonal antibody specific to the HA epitope (α-HAHA). The cell lysates and immunoprecipitates (IP) were fractionated by SDS-PAGE and analyzed by immunoblotting (IB) using mouse monoclonal antibodies specific to either the HA or Myc epitopes (α-HAHA or α-Myc, respectively). The two immunoreactive bands visualized in the NHE1HA blots represent the immature core-glycosylated (cg) and mature fully glycosylated (fg) forms of the exchanger. Data shown are representative of three separate experiments.

FIGURE 1. N-Myristoylation and Ca2+ binding-defective mutants of CHP3 form a complex with NHE1 in transfected cells. Chinese hamster ovary AP-1 cells stably expressing NHE1HA were transiently transfected with either wild-type or mutant constructs of CHP3myc that are defective in either N-myristoylation (G2A) or calcium-binding (D123A). Twenty four hours post-transfection, cell lysates were prepared, and NHE1HA-containing protein complexes were immunoprecipitated with a rabbit polyclonal antibody specific to the HA epitope (α-HAHA). The cell lysates and immunoprecipitates (IP) were fractionated by SDS-PAGE and analyzed by immunoblotting (IB) using mouse monoclonal antibodies specific to either the HA or Myc epitopes (α-HAHA or α-Myc, respectively). The two immunoreactive bands visualized in the NHE1HA blots represent the immature core-glycosylated (cg) and mature fully glycosylated (fg) forms of the exchanger. Data shown are representative of three separate experiments.

To further establish the physical association between NHE1HA and the various CHPmyc constructs, their respective subcellular distributions were compared using dual immunolabeling and fluorescence confocal microscopy. Previous studies (25) showed that when coexpressed in AP-1 cells, NHE1 and CHP3 colocalize at the cell surface. However, when CHP3 is expressed in AP-1 cells devoid of NHE1 or coexpressed with mutant forms of NHE1 that do not interact with CHP3, CHP3 fails to accumulate at the cell surface but instead is diffusely distributed throughout the cytoplasm (25). As shown in Fig. 2A, WT as well as N-myristoylation (G2A) and Ca2+ binding (D123A)-defective mutants of CHP3myc colocalized with NHE1HA at the plasma membrane, although there was an increased tendency for the D123A mutant to also accumulate...
intracellularly (Fig. 2A). Consistent with this visual assessment, quantitative statistical analyses of the colocalization of the respective fluorophores indicated strong associations between NHE1HA and CHP3myc-WT or -G2A (Pearson’s correlation coefficient \( r = 0.87 \) and 0.68, respectively) and medium correlation between NHE1HA and CHP3myc-D123A \( (r = 0.36) \). However, in the absence of NHE1, all three forms of CHP3 were distributed more diffusely throughout the cell (Fig. 2B). These results demonstrate that \( N \)-myristoylation and \( Ca^{2+} \) binding of CHP3 are not essential for the interaction with NHE1.

Previous studies by Pang et al. (17) suggested that \( Ca^{2+} \) binding to the two functional EF-hand domains of CHP1 greatly influenced its interaction with NHE1. Furthermore, Gutierrez-Ford et al. (16) suggested that CHP3 likely binds \( Mg^{2+} \) in its resting state. However, upon cellular stimulation that increases intracellular \( Ca^{2+} \), the \( Mg^{2+} \) would be displaced by \( Ca^{2+} \), which then induces a conformational change in CHP3 to an “active” state. To investigate whether the binding of \( Ca^{2+} \) to CHP3 influences its interaction with NHE1, we performed a coimmunoprecipitation assay in the absence or presence of 0.1 mM \( Ca^{2+} \). AP-1 cells were transiently cotransfected with NHE1HA and either the WT or mutant D123A construct of CHP3myc. After 48 h, cells lysates were prepared in RIPA buffer supplemented with both 1 mM \( MgCl_2 \) and 1 mM EDTA or with 0.1 mM \( CaCl_2 \). Cell lysates were incubated with a rabbit polyclonal anti-Myc antibody, and the resulting CHP3myc-containing immunoprecipitates were subject to SDS-PAGE and immunoblotting to visualize the extent of association with NHE1HA.

**FIGURE 2.** \( N \)-Myristoylation (G2A) and \( Ca^{2+} \)-binding (D123A)-defective mutants of CHP3 colocalize with NHE1HA at the plasma membrane. Immunofluorescence confocal microscopy of AP-1 cells stably expressing NHE1HA and transiently transfected with either wild-type or mutant forms (G2A, D123A) of CHP3myc \( (A) \) or AP-1 cells transiently transfected with the CHP3myc constructs in the absence of NHE1 \( (B) \). Subcellular distribution of NHE1HA was visualized using mouse monoclonal antibodies specific to the HA epitope followed by labeling with a goat anti-mouse secondary antibody conjugated to AlexaFluor\textsuperscript{TM}488. CHP3myc distribution was identified through a primary rabbit polyclonal antibody specific to the Myc epitope followed by a secondary goat anti-rabbit antibody conjugated to AlexaFluor\textsuperscript{TM}568. Overlapping signals in the merged images are shown in yellow. Data are representative of between two and four independent experiments. Scale bars at the bottom right of each panel represent 10 \( \mu \)m.
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As shown in Fig. 3, the amount of NHE1HA that forms a complex with the CHP3myc, in the presence of Ca\(^{2+}\), was \(\sim 35 \pm 8\%\) higher compared with conditions containing nominal levels of Ca\(^{2+}\). In the case of the Ca\(^{2+}\)-binding-deficient CHP3mycD123A, there was no detectable difference in its association with NHE1 in the absence or presence of Ca\(^{2+}\). This result indicates that Ca\(^{2+}\) is not essential for the binding of CHP3 to NHE1 but does promote a stronger interaction.

**N-Myristoylation and Ca\(^{2+}\): Binding of CHP3 Are Required for Optimal NHE1 Activity**—Previously, we showed that expression of CHP3 increases NHE1 activity by enhancing its biosynthetic maturation and its stability at the plasma membrane (25). To determine whether N-myristoylation or Ca\(^{2+}\) binding of CHP3 influences the production and function of NHE1, AP-1 cells that stably express NHE1HA were transiently transfected with WT NHE1HA and either empty vector or different constructs of CHP3myc (WT, G2A, or D123A), and the cell lysates were prepared at time points up to 72 h following transfection to assess the expression profiles of NHE1HA and CHP3myc. As shown in Fig. 5, NHE1HA alone showed a transitory increase in both core and fully glycosylated forms that reached maximal levels at \(\sim 24\) h, with the bulk being primarily core-glycosylated. By comparison, although NHE1HA cotransfected with WT CHP3myc also showed a similar temporal increase in expression of both the core and fully glycosylated NHE1HA, there was a much greater accumulation of the fully glycosylated relative to the core-glycosylated form, consistent with previous findings (25). Notably, loss of N-myristoylation or Ca\(^{2+}\) binding of CHP3myc did not impair the rate of processing of newly synthesized NHE1HA.

To directly verify that CHP3-mediated increases in fully glycosylated NHE1 protein and transport activity indeed reflect its accumulation at the cell surface, as implied by the data presented in Fig. 4, plasmalemmal NHE1 levels were directly measured using a cell surface biotinylation assay (35). To this end, AP-1 cells were transiently cotransfected with a fixed amount of NHE1HA and an increasing ratio of CHP3myc, to empty vector (pCMV). The cells were also cotransfected with an expression of both immature core-glycosylated and mature fully glycosylated forms of NHE1HA (Fig. 4A), particularly the latter. Because we previously demonstrated that the bulk of fully glycosylated NHE1 resides at the plasma membrane, whereas the core-glycosylated species resides intracellularly (25, 34), we measured the protein levels of fully glycosylated NHE1HA by immunoprecipitation and correlated it with NHE1 activity as a function of CHP3myc expression. As shown in Fig. 4, B and C, WT CHP3myc-mediated increases in the abundance of fully glycosylated NHE1 closely paralleled, albeit to a higher extent, the increases in NHE1 activity \((i.e. \sim 2.2\)-fold versus \(\sim 1.6\)-fold, respectively). The reason for the lack of tighter correlation between NHE1 protein abundance and activity is unclear, but it may reflect differences in the sensitivities of the respective methodologies. Alternatively, it could be that not all of the fully glycosylated NHE1 molecules, as measured by densitometry, have yet to reach the cell surface. Similarly, both the N-myristoylated and Ca\(^{2+}\)-binding-defective mutants of CHP3myc, which were produced at levels equivalent to WT CHP3myc, also increased the abundance and activity of NHE1HA, but to lesser extents than WT CHP3myc. This suggests that these structural elements, although not critical for the ability of CHP3 to bind and promote the maturation and activity of NHE1, do contribute to its potency.

**N-Myristoylation and Ca\(^{2+}\): Binding of CHP3 Do Not Influence the Rate of Maturation of Newly Synthesized NHE1**—Previously, we showed that CHP3 stimulates the post-translational maturation, cell surface accumulation, and stability of NHE1 (25). The new results presented in Fig. 4 suggested that N-myristoylation and Ca\(^{2+}\) binding of CHP3 are involved in optimizing cell surface NHE1 abundance and activity. To further investigate the mechanistic basis for this observation, we first investigated the oligosaccharide maturation of NHE1 by using a transient transfection approach to monitor newly synthesized exogenous proteins. Thus, AP-1 cells were transiently transfected with NHE1HA and either empty vector or different constructs of CHP3myc (WT, G2A, or D123A), and cell lysates were fractionated by SDS-PAGE and immunoblotting (IB). NHE1HA was visualized with a mouse monoclonal antibody specific to the HA epitope \((\alpha\text{-HA})\), cg, core-glycosylated; fg, fully glycosylated. B, intensities of the immunoreactive signals for NHE1 presented in A were measured by densitometry using ImageJ \(^{\circ}\) and normalized to the signals obtained in the absence of Ca\(^{2+}\) (mean ± S.E., \(n = 3\)).
plasmid that constitutively expresses green fluorescent protein (pGFP) as a control for transfection efficiency. Forty-eight hours post-transfection, plasma membrane proteins were selectively extracted for analysis of NHE1 abundance by immunoblotting. As illustrated in Fig. 6, the fully glycosylated form of NHE1HA was the predominant species detected at the cell surface, and its increased abundance correlated with enhanced expression of wild-type as well as mutated forms of CHP3myc. Hence, N-myristoylation and Ca²⁺ binding of CHP3 are not essential elements for CHP3-mediated trafficking of NHE1 to the cell surface, consistent with the microscopy results presented in Fig. 2.

**N-Myristoylation and Ca²⁺ Binding of CHP3 Do Not Influence the pHᵢ Sensitivity of NHE1**—Previous studies have indicated that Ca²⁺ binding to the CHPI isoform was an important determinant of the pHᵢ sensitivity of NHE1 (17). To examine whether this might also apply to CHP3, the H⁺ sensitivity of NHE1HA was measured in the absence or presence of WT and mutant forms of CHP3myc. To facilitate measurements of NHE1 pHᵢ sensitivity, AP-1 cell lines were generated that stably express NHE1HA alone or in combination with the WT, G2A, or D123A variants of CHP3myc. In each case, the total abundance of NHE1HA in cells expressing the CHP3myc variants was enhanced compared with cells expressing NHE1 alone (Fig. 7A), consistent with the transient transfection assays. The pHᵢ profile of NHE1 in the various stable cell lines was then assessed by measuring the initial rates of amiloride-inhibitable ²²Na⁺ influx (pmol/min/mg total cellular protein) following an acute intracellular acid load induced by pre-pulsing with NH₄Cl, as described under “Experimental Procedures.” To facilitate comparison, the activity data were normalized to AP-1/NHE1HA cells that do not express CHP3 (± 2 pmol/min/mg protein) and represented as relative changes in NHE1 activity. Values represent the mean ± S.E. of three experiments, each performed in triplicate.

**N-Myristoylation and Ca²⁺ Binding of CHP3 Are Required for Cell Surface Stability of NHE1**—Although N-myristoylation and Ca²⁺ binding of CHP3myc do not seem to be required for the CHP3-mediated enhancement of post-translational processing of NHE1, the cell surface accumulation and transport activities of the exchanger in AP-1 cells expressing N-myristoylation- or Ca²⁺ binding-defective CHP3myc did not affect the H⁺ affinity of the exchanger.

**N-Myristoylation and Ca²⁺ Binding of CHP3**

- Do not influence the pHᵢ sensitivity of NHE1.
- Are required for cell surface stability of NHE1.

These findings highlight the importance of N-myristoylation and Ca²⁺ binding for effective trafficking and surface localization of NHE1 in AP-1 cells.
CNP3myc is also known to stabilize NHE1HA at the plasma membrane. To test the importance of N-myristoylation and Ca\textsuperscript{2+} binding of CNP3myc in this process, the half-life of fully glycosylated NHE1HA alone or together with each of the CNP3myc variants (WT, G2A, and D123A) was measured using a biotinylation pulse-chase assay as described previously (25). Briefly, plasmalemmal proteins of AP-1 cell lines stably expressing NHE1HA alone or in combination with the WT, G2A, or D123A variants of CNP3myc were covalently linked to biotin using the membrane-impermeant reagent sulfo-NHS-SS-biotin. Following removal of excess reagent, the cells were incubated in regular culture media over a 48-h period. At several time points during this period, the biotylated proteins were extracted from the cell lysates using NeutrAvidin™-Sepharose beads, fractionated by SDS-PAGE, and analyzed by immunoblotting. Expression of cell surface fully glycosylated NHE1HA for the various time points was measured by densitometry, normalized to maximum NHE1HA expression at 0 h, and then plotted as a function of time. As shown in Fig. 8, A and B, the half-life of biotinylated, fully glycosylated NHE1HA was ~3.8-fold higher in the presence of WT CNP3myc than in its absence (14.5 ± 3.6 h versus 3.8 ± 0.6 h, respectively). However, the half-life of NHE1HA in cells coexpressing either the N-myristoylation-defective (G2A) or the Ca\textsuperscript{2+} binding-defective (D123A) mutant of CNP3 was considerably reduced (6.1 ± 1.1 and 4.8 ± 0.8 h, respectively) compared with WT CNP3myc and, although intermediate, more closely paralleled that of cells expressing NHE1HA alone (3.8 ± 0.6 h). As a side note, we observed that the total cellular levels of NHE1HA and CNP3myc (WT, G2A, D123A) increased as a function of time in culture. Although the reason for this is unclear, this may relate to the constitutive overproduction of these exogenous proteins when driven transcriptionally by a strong "unregulated" viral pro-
DISCUSSION

CHP3 is the most recently identified member of a family of N-myristoylated, EF-hand Ca\(^{2+}\)-binding proteins that bind to the cytoplasmic juxtamembrane C termini of plasma membrane-type NHEs and regulate their activities in unique, albeit overlapping, ways (18, 20, 25, 26, 38). Previously, we reported that CHP3 promotes the oligosaccharide maturation and trafficking of NHE1 along the exocytic pathway as well as its half-life and transport activity at the cell surface (25). To better understand the molecular mechanisms underlying this regulation, we examined the potential relevance of the N-myristoylation site and single Ca\(^{2+}\)-binding domain of CHP3 to this process using a site-directed mutagenesis approach.

Here, we show that N-myristoylation or Ca\(^{2+}\) binding are neither essential for the interaction of CHP3 with NHE1 nor are they necessary for CHP3-mediated oligosaccharide maturation and trafficking of the transporter to the plasma membrane. However, disruption of either structural element markedly diminished the ability of CHP3 to enhance the steady-state abundance and corresponding activity of NHE1 at the plasma membrane to a similar extent, effects that were not further altered by a double mutation. This change correlated with a significant reduction in the cell surface stability of the exchanger.

The observation that the N-myristoylation and Ca\(^{2+}\) binding-defective mutants of CHP3 were able to enhance the posttranslational maturation and surface delivery of NHE1 at rates comparable with WT CHP3 suggests that other structural domains of CHP3 to this process using a site-directed mutagenesis approach.

Here, we show that N-myristoylation or Ca\(^{2+}\) binding are neither essential for the interaction of CHP3 with NHE1 nor are they necessary for CHP3-mediated oligosaccharide maturation and trafficking of the transporter to the plasma membrane. However, disruption of either structural element markedly diminished the ability of CHP3 to enhance the steady-state abundance and corresponding activity of NHE1 at the plasma membrane to a similar extent, effects that were not further altered by a double mutation. This change correlated with a significant reduction in the cell surface stability of the exchanger.

The observation that the N-myristoylation and Ca\(^{2+}\) binding-defective mutants of CHP3 were able to enhance the posttranslational maturation and surface delivery of NHE1 at rates comparable with WT CHP3 suggests that other structural domains of CHP3 are engaged in this process upon binding NHE1. Although the precise mechanism is unknown, this could conceivably involve CHP3-dependent recruitment of components implicated in protein trafficking. Such a suggestion is not
without precedent, as previous studies (39) have shown that its paralog CHP1/p22 interacts with the multifunctional protein GAPDH that facilitates the binding of CHP1/p22 to microtubules independent of its association with microsomal membranes (and associated cargo). Whether GAPDH also fulfills a similar role for CHP3 remains to be determined. Notwithstanding, it would appear that N-myristoylation and Ca\(^{2+}\)/H\(_{11001}\) binding of CHP3 are not required for the early events in NHE1 processing but are required at the plasma membrane to increase the residency time of the transporter.

At present, there is little tertiary structure information available for CHP3. However, the finding that the individual as well as double mutations yielded identical behavior suggests that these distant sites are mechanically and functionally linked in a manner that would be consistent with a Ca\(^{2+}\)-myristoyl switch mechanism, as has been described for some N-myristoylated Ca\(^{2+}\)-binding proteins such as recoverin and guanylate cyclase-activating proteins (36, 40, 41). In this regard, we observed that although a functional Ca\(^{2+}\)-binding site was not required for the assembly of an NHE1-CHP3 complex at nominal Ca\(^{2+}\) concentrations, elevation of Ca\(^{2+}\) levels to micromolar levels significantly increased the in vitro binding strength of wild-type CHP3 to NHE1, effects that were abrogated by mutating the Ca\(^{2+}\)-binding site. Previous analyses by Gutierrez-Ford et al. (16) showed that recombinant CHP3 binds a single Ca\(^{2+}\) ion in the third predicted EF-hand domain with an apparent affinity (K\(_d\)) of 0.8 M, a value that is within the physiological range for sensing changes in intracellular Ca\(^{2+}\). Furthermore, by measuring intrinsic tryptophan fluorescence, they detected a small but significant Ca\(^{2+}\)-dependent conformational change in CHP3. Although the precise meaning of this structural perturbation remains to be determined, it could conceivably enhance the affinity of CHP3 for NHE1 while at the same time exposing the N-myristoylation site, thereby facilitating the attachment of CHP3 to the inner leaflet of the membrane. In principle, this might strengthen the retention of the NHE1-CHP3 complex at the cell surface. Furthermore, such an arrangement could also act to align the juxtamembrane cytoplasmic C terminus of NHE1 in close proximity to the inner membrane surface that may be important for transport activity. Indeed, previous studies (34) have shown that the CHP-binding site of NHE1 is situated between two positively charged amino acid clusters that form an electrostatic interaction with phosphatidylinositol 4,5-bisphosphate embedded in the inner leaflet of the plasma membrane, which could further strengthen the orientation of that segment of the C terminus of NHE1 in tight

FIGURE 8. N-Myristoylation and Ca\(^{2+}\) binding of CHP3 enhance cell surface stability of NHE1. A, AP-1 cells stably expressing NHE1\(_{1\alpha}\) or stably coexpressing NHE1\(_{1\alpha}\) and individual variants of CHP3\(_{\text{myc}}\) (WT, G2A, and D123A) were subject to cell surface biotinylation, as described under "Experimental Procedures." The cells were returned to growth media at 37 °C, and then cell lysates were prepared at varying times over a 48-h period. At each time point, a small fraction of the cell lysates was removed for immunoblotting, and the remainder was incubated with NeutrAvidin-Sepharose beads to extract the biotinylated proteins. Total cellular levels of core-glycosylated (cg) and fully (fg) glycosylated NHE1\(_{1\alpha}\) and CHP3\(_{\text{myc}}\), as well as levels of surface-biotinylated, fully glycosylated NHE1\(_{1\alpha}\) were monitored as a function of time by SDS-PAGE and immunoblotting, as described in the legend to Fig. 4. It was noted that occasionally a small amount of the core-glycosylated NHE1\(_{1\alpha}\) was detected in the cell surface biotinylated fraction, possibly indicating contamination from intracellular compartments. However, when the blots were stripped and reprobed for intracellular GAPDH, no signal was detected (data not shown). This suggests that a minor fraction of the core-glycosylated NHE1 can traffic to the plasma membrane, perhaps as a consequence of overexpression. B, data represent densitometric analysis of the cell surface fully glycosylated NHE1\(_{1\alpha}\) presented in A, normalized as a percentage of its maximal abundance at time 0 h and plotted as a function of time. Values represent the mean ± S.E. of three experiments. Error bars smaller than the symbol are absent.
The functional relevance of \( N \)-myristoylation and \( Ca^{2+} \) binding of CHP3 has also been examined in the context of the NHE1 complex. Similar to CHP3, mutation of the \( N \)-myristoylation site in CHP1 did not alter the binding, membrane trafficking, or pH sensitivity of NHE1 (17). However, the contributions of \( Ca^{2+} \) binding to CHP1 function appear to differ markedly from those of CHP3. Unlike CHP3, CHP1 contains two very high affinity \( Ca^{2+} \)-binding domains (EF3 and EF4), which increase their avidity for \( Ca^{2+} \) by \( \sim 45 \)-fold (\( K_d \sim 90 \rightarrow 2 \) nm) upon the formation of a complex with the CHP-binding domain of NHE1 (17, 44). Given that resting intracellular \( Ca^{2+} \) concentrations are estimated to be \( \sim 100 \) nm, CHP1 would bind \( Ca^{2+} \) constitutively. Single mutations that prevent \( Ca^{2+} \) binding to either EF3 or EF4, while not impairing its binding to NHE1, caused a significant reduction in its pH sensitivity (17). This shift to more acidic values reduced basal NHE1 activity and markedly impaired its activation by various extracellular stimuli. Moreover, disruption of both \( Ca^{2+} \)-binding sites abolished the interaction of CHP1 with NHE1, resulting in the accumulation of the \( Ca^{2+} \)-binding-defective CHP1 in the cytoplasm. Thus, Pang et al. (17) concluded that the two \( Ca^{2+} \)-binding domains of CHP1 most likely do not act collectively as a \( Ca^{2+} \) sensor but rather as important structural elements that help to control the pH sensing of NHE1. This behavior is in contrast to that of the \( Ca^{2+} \)-binding-defective CHP3, which retains its ability to bind to NHE1 without altering its pH sensitivity. Although the molecular basis for this isoform difference is unclear, CHP3 possesses three other helix-loop-helix structural domains in addition to its one functional EF-hand \( Ca^{2+} \)-binding domain that are more divergent in sequence compared with CHP1 and CHP2, and therefore they might be uniquely arranged to better stabilize the protein through intramolecular EF-hand pairing (45). In the case of CHP1 (and possibly CHP2), the mutations that prevent \( Ca^{2+} \) binding may impart a more severe structural deformation that prevents further interactions with its remain-
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...ing helix-loop-helix structures needed to interact with NHE1. Thus, with respect to the NHE1-CHP1 complex, the role of the N-myristoylation remains unclear, but it does not appear to be functionally coupled to the Ca\(^{2+}\)-binding domains. In summary, this study provides further insight into the roles of N-myristoylation and Ca\(^{2+}\) binding of CHP3 and its regulation of NHE1. CHP3 regulates the processing and stability of cell surface NHE1, and although N-myristoylation and Ca\(^{2+}\) binding are not required for binding and for maturation of the exchanger, they play a crucial role in stabilizing NHE1 at the plasma membrane to promote optimal Na\(^{+}/\)H\(^{+}\) exchange activity.

Acknowledgment—We kindly thank Kimberley Young for assistance with the quantitative statistical analyses of the colocalization image data presented in this paper.

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