Casein Kinase 2 Binds to the C Terminus of Na⁺/H⁺ exchanger 3 (NHE3) and Stimulates NHE3 Basal Activity by Phosphorylating a Separate Site in NHE3

Rafiquel Sarker,* Mads Grønberg,^{†‡} Boyoung Cha,* Sachin Mohan,* Yueping Chen,* Akhilesh Pandey,[†] David Litchfield,[§] Mark Donowitz,*^{||¶} and Xuhang Li*[¶]

*Division of Gastroenterology and Hepatology, Department of Medicine, [†]Institute of Genetic Medicine, ^{||}Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205; and [§]Department of Biochemistry, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada, N6A 5C1

Submitted January 9, 2008; Revised May 28, 2008; Accepted June 22, 2008
Monitoring Editor: M. Bishr Omary

Na⁺/H⁺ exchanger 3 (NHE3) is the epithelial-brush border isoform responsible for most intestinal and renal Na⁺ absorption. Its activity is both up- and down-regulated under normal physiological conditions, and it is inhibited in most diarrheal diseases. NHE3 is phosphorylated under basal conditions and Ser/Thr phosphatase inhibitors stimulate basal exchange activity; however, the kinases involved are unknown. To identify kinases that regulate NHE3 under basal conditions, NHE3 was immunoprecipitated; LC-MS/MS of trypsinized NHE3 identified a novel phosphorylation site at S⁷¹⁹ of the C terminus, which was predicted to be a casein kinase 2 (CK2) phosphorylation site. This was confirmed by an in vitro kinase assay. The NHE3-S719A mutant but not NHE3-S719D had reduced NHE3 activity due to less plasma membrane NHE3. This was due to reduced exocytosis plus decreased plasma membrane delivery of newly synthesized NHE3. Also, NHE3 activity was inhibited by the CK2 inhibitor 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole DMAT when wild-type NHE3 was expressed in fibroblasts and Caco-2 cells, but the NHE3-S⁷¹⁹ mutant was fully resistant to DMAT. CK2 bound to the NHE3 C-terminal domain, between amino acids 590 and 667, a site different from the site it phosphorylates. CK2 binds to the NHE3 C terminus and stimulates basal NHE3 activity by phosphorylating a separate single site on the NHE3 C terminus (S⁷¹⁹), which affects NHE3 trafficking.

INTRODUCTION

Neutral NaCl absorption is a small intestinal and colonic absorptive process in which a brush-border (BB) Na⁺/H⁺ exchanger is coupled, at least functionally, to a BB Cl[−]/HCO₃[−] exchanger. In intestinal Na⁺ absorptive cells, the Na⁺/H⁺ exchanger involved is SLC9A3 (NHE3), and the anion exchanger is either SLC26A3 or 6 (DRA or PAT-1) (Lamprecht *et al.*, 2002; Zachos *et al.*, 2005). This process is highly regulated and a defining characteristic is that it is set at an intermediate level during the period between meals (called basal conditions), which allows both inhibition and stimulation to occur as part of digestion (Moe, 1999; Zachos *et al.*, 2005; Donowitz and Li, 2007).

NHE3 is the highly regulated part of the neutral NaCl absorptive process. In all cells in which it has been studied,

NHE3 regulation mimics the regulation of neutral NaCl absorption, being active under basal conditions and being both inhibited and stimulated in cell models by various mimics of digestive physiology. NHE3 is phosphorylated under both basal and regulated conditions (Yip *et al.*, 1998; Wiederkehr *et al.*, 1999; Wang *et al.*, 2005). Identified sites of phosphorylation in the NHE3 C terminus include amino acids S⁵⁵⁴ and S⁶⁰⁷ (rabbit NHE3), which occurs both under basal conditions and increases after cAMP, and also S⁶⁶³, which is phosphorylated by serum and glucocorticoid-inducible kinase (SGK1) in acute stimulation by glucocorticoids (Wang *et al.*, 2005).

The function of phosphorylation of NHE3 under basal conditions is not known, but cAMP-increased phosphorylation leads to NHE3 inhibition by steps that involve an initial inhibition of turnover number and then reduced plasma membrane expression due first to increased endocytosis and later reduced endocytic recycling (Moe, 1999). Phosphatase inhibitors also affect NHE3. Okadaic acid (PP1 and 2A inhibitor) stimulates NHE3 activity and genistein (Tyr kinase inhibitor) inhibits NHE3 (Levine *et al.*, 1995). These results suggest that both S/T and Y phosphorylation stimulate NHE3 but do not indicate whether there are changes in phosphorylation of NHE3 or whether NHE3 activity is affected by changes in phosphorylation of regulatory proteins, although NHE3 has not been shown to be Tyr phosphorylated (Yip *et al.*, 1998).

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-01-0019>) on July 9, 2008.

[¶] These authors contributed equally to this work.

[‡] Present address: Department of Neurobiology, Max-Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany.

Address correspondence to: Mark Donowitz (mdonowitz@jhmi.edu) or Xuhang Li (xuhang@jhmi.edu).

The current studies were based on the hypothesis that understanding of intestinal Na^+ absorption requires understanding of both basal and regulated NHE3 activity at the level of the protein kinases involved. This study used a proteomics approach to identify and characterize the functional effects of a unique NHE3 phosphorylation site (S⁷¹⁹) that was subsequently identified as phosphorylated by casein kinase 2 (CK2).

MATERIALS AND METHODS

Chemicals, Reagents, and Antibodies

Reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. 4,5,6,7-Tetrabromo-2-azabenzimidazole (TBB) and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) were from Calbiochem (San Diego, CA). QuikChange site-directed mutagenesis kit and *Pfu* polymerase were from Stratagene (La Jolla, CA). EZ-Link Sulfo-NHS-SS-biotin and Sulfo-NHS-acetate were from Pierce Chemical (Rockford, IL). Glutathione-Sepharose 4B beads were from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). Restriction endonucleases were from New England Biolabs (Ipswich, MA). 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Invitrogen (Carlsbad, CA). P81 phosphocellulose papers were from Whatman (Maddstone, United Kingdom). Monoclonal mouse antibodies to the hemagglutinin (HA) epitope was from Covance Research Products (Princeton, NJ). Rabbit polyclonal anti-CK2 α , and CK2 β antibodies, were from Abcam (Cambridge, United Kingdom). DNA primers were from Operon Biotechnologies (Huntsville, AL). Peptides were synthesized at the Johns Hopkins Synthesis and Sequencing Facility (Baltimore, MD).

Mass Spectrometry Analysis and Database Searching

In-Gel Digestion. Immunoprecipitated NHE3 was first resolved by one-dimensional (1D) SDS-polyacrylamide gel electrophoresis (PAGE), and protein bands were visualized by Coomassie Blue staining. A protein band corresponding to NHE3 was excised from the gel and washed two times in MilliQ water (Millipore, Billerica, MA) and water:acetonitrile (1:1, vol/vol) for 15 min at room temperature. After washing the liquid was removed and replaced by 100% acetonitrile to shrink the gel pieces. After the gel pieces turned white, the acetonitrile was removed and replaced by 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 45 min at 56°C to reduce Cys. The liquid was then removed and replaced by 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min at room temperature in the dark to alkylate the Cys. The gel pieces were then dehydrated in 100% acetonitrile and rehydrated in digestion buffer containing 12.5 ng/ μl trypsin (modified sequence grade; Promega, Madison, WI) in 50 mM ammonium bicarbonate and incubated on ice for 45 min (Gronborg *et al.*, 2004). The digestion buffer was then aspirated and replaced by 50 mM ammonium bicarbonate without trypsin and incubated overnight at 37°C. After digestion the supernatant was removed and the remaining peptides were extracted by incubating the gel pieces in 5% formic acid for 15 min and then adding the same volume of 100% acetonitrile for 15 min. The extraction was repeated twice and all three fractions were pooled and dried down in a vacuum centrifuge and resuspended in 10 μl of 5% formic acid.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). After tryptic digestion of NHE3, the peptides were analyzed by nanoLC-MS/MS. The samples were loaded onto a 75- μm fused silica precolumn packed with 10- μm C₁₈ ODS-A (YMC, Kyoto, Japan) for washing and desalting in 95% mobile phase A (H₂O with 0.4% acetic acid and 0.005% heptafluorobutyric acid, vol/vol) and 5% mobile phase B (90% acetonitrile, 0.4% acetic acid, and 0.005% heptafluorobutyric acid in water, vol/vol). After washing and desalting, the sample was eluted from the precolumn by a linear gradient of 90% mobile phase A to 60% mobile phase A onto a 75- μm fused silica analytical column packed with Vydac C₁₈ resin. The peptides were eluted over a gradient of 34 min. The spectra were acquired on a QTOF API-US (Waters-Micromass, Manchester, United Kingdom). All data were obtained in positive ion-mode, and the data were analyzed using MassLynx 4.0 software (Waters-Micromass).

Database Searching. Database searching was done using the Mascot Search engine (Matrix Science, London, United Kingdom). The data were searched against the NCBI database with a mass accuracy of 0.3 Da for the parent ion (MS) and 0.3 for the fragment ions (MS/MS). The peptides were constrained to be tryptic with a maximum of two missed cleavages. Fixed modifications were carbamidomethylation of Cys, whereas oxidation of Met and phosphorylation of Ser were considered as variable modifications.

Cell Culture, Plasmid Construction, and Transfection

PS120 fibroblasts, which lack all endogenous plasma membrane NHEs, were used for stable expression of rabbit NHE3-WT, NHE3-S719A, and NHE3-S719D, with either a triple-HA epitope tag at the N terminus (Murtazina *et al.*, 2006) or a C-terminal vesicular stomatitis virus glycoprotein (VSV-G) epitope tag (Levine *et al.*, 1995). The NHE3-S719A and NHE3-S719D mutations were made using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The template for mutagenesis was the pcDNA3.1/Neomycin⁺ vector (Invitrogen) containing rabbit 3HA-NHE3. All PS120 cell lines were grown in DMEM supplemented with 25 mM NaHCO₃, 10 mM HEPES, 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 400 $\mu\text{g}/\text{ml}$ G418 (Neomycin), and 10% fetal bovine serum in a 5% CO₂, 95% O₂ incubator at 37°C. Caco-2/bbe cell line originally derived from a human adenocarcinoma was obtained from M. Mooseker (Yale University, New Haven, CT) and grown in DMEM containing 25 mM NaHCO₃ supplemented with 0.1 mM nonessential amino acids, 10% fetal bovine serum, glutamine, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin, pH 7.4, in 5% CO₂ atmosphere at 37°C. Stably transfected PS120 cells were grown on glass coverslips up to 60–70% confluence and studied after serum starvation for 3–6 h. PS120/HA-NHE3 and HA-NHE3-S719A cell lines were selected for Na⁺/H⁺ exchange activity (3–4 passages) by exposing cells to an acid load as described previously (Levine *et al.*, 1993). Caco-2/bbe cells were grown to confluence on small pieces of polycarbonate membranes glued to plastic coverslips (0.4- μm pore size, Corning; called “filter slips”) and polarized cells were used ~14 d after reaching confluence.

Adenoviral Construct/Infection

Tripled HA-tagged rabbit NHE3 was engineered into Adeno-viral shuttle vector ADLOX.HTM under a cytomegalovirus promoter. Caco-2/bbe cells were treated with 6 mM EGTA in serum-free Caco-2 medium for 2 h at 37°C before viral infection to allow virus exposure to the basolateral surface. Appropriate amounts of viral particles were diluted (10⁹–10¹⁰ particles/ml) in serum-free Caco-2 medium and added to upper and lower chambers of EGTA-treated cells. Cells were infected by incubating at 37°C for 6 h and then replaced with normal growth medium. For transport assays or Western analyses, cells were used ~48–60 h after infection; infection was typically day 11–12 after confluence and study was day 14. Generally, viral production was generated by transfection of CRE8 cells with the expressing constructs using Lipofectamine 2000 (Invitrogen), followed by infection of the cells with helper virus (ψ 5). Then, the crude adenoviruses were propagated by reinfection of CRE8 cells. Preparation of purified adenovirus with high titer was performed by CsCl gradient centrifugations. Viral titers were determined by a colorimetric method, following 1) the A₃₂₀ was below 0.02 and 2) the A₂₆₀/A₂₈₀ was between 1.2 and 1.3. Particle numbers were calculated by (A₂₆₀ value) $\times$ (1.1 $\times$ 10¹²) $\times$ dilution.

Measurement of Na⁺/H⁺ Exchange Activity

Na⁺/H⁺ exchange activity in PS120 and Caco-2 cells expressing HA-NHE3 was determined fluorometrically using the intracellular pH-sensitive dye BCECF, with PS120 cells grown to 60–70% confluence on glass coverslips (Levine *et al.*, 1995) and Caco-2/bbe cells polarized on 0.4- μm polycarbonate membranes; they were studied ~14 d after confluence as described previously (Janecki *et al.*, 1998). Cells were loaded with 10 μM BCECF-AM in Na⁺/NH₄Cl solution (98 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM glucose, 20 mM HEPES, and 40 NH₄Cl, pH 7.4) for 20 min at 37°C. During the dye loading and NH₄Cl prepulse, cells were treated with CK2 inhibitors, unless timing is described otherwise. The cells were initially perfused with TMA⁺ solution (130 mM tetramethylammonium chloride, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM glucose, and 20 mM HEPES, pH 7.4), resulting in stable acidification of the cells. Then, Na⁺ solution (138 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM glucose, and 20 mM HEPES, pH 7.4) was perfused. CK2 inhibitor was also present in TMA and Na⁺ solutions. To calibrate the relationship between the emission (530 nm) from the dual excitation (F₄₉₀ and 440) and pH_i, the K⁺/nigericin method was used. As described previously (Levine *et al.*, 1995), Na⁺/H⁺ exchange rates (H⁺ efflux) were calculated as the product of Na⁺-dependent change in pH_i and the buffering capacity at each pH_i and were analyzed using the nonlinear regression data analysis program Origin, which allows fitting of data to a general allosteric model described by the Hill equation ($v = V_{\text{max}} \cdot [\text{S}]^{n_{\text{app}}} / K' + [\text{S}]^{n_{\text{app}}}$), where v is velocity, $[\text{S}]$ is the substrate concentration, n_{app} is the apparent Hill coefficient, and K' is the affinity constant ($K'(\text{H}^+)_i$), with estimates for V_{max} and $K'(\text{H}^+)_i$ and their respective errors (SE), as well as fitting to a hyperbolic curve. Data from each coverslip were calculated and analyzed as described above. For each independent experiment, results from all coverslips for each condition were analyzed together and represent an $N = 1$. To measure the Na⁺/H⁺ exchange activity in Caco-2/bbe cells, the cells were grown as polarized monolayers on pieces of polycarbonate membranes (0.4- μm pore size) attached over an aperture in rectangular plastic coverslips (“filter slip”), and cellular Na⁺/H⁺ exchange activity was determined fluorometrically by loading the intracellular pH-sensitive dye BCECF-AM for 60 min (Janecki *et al.*, 1998). Filter slips were then mounted in a cuvette, placed

in the fluorometer (Photon Technology International, Lawrenceville, NJ), and perfused at both apical and basolateral monolayer surfaces with TMA medium to allow rapid intracellular acidification. The TMA⁺ medium was subsequently replaced with Na⁺ medium at the apical monolayer surface, whereas TMA perfusion continued basally. Initial rates of Na⁺-dependent intracellular alkalization (efflux of H⁺, in micromolar per second) were calculated for a given pH_i; ~1 min of the initial rate of intracellular alkalization was analyzed as $\Delta\text{pH}/\Delta\text{T}$. Means $\pm$ SE were determined from at least three experiments.

Expression and Purification of Fusion Proteins

Recombinant glutathione S-transferase (GST-CK2 α) was made in the (pGEX) vector. The 6x His-tagged fusion proteins made in the pET30a vector included four fragments of the NHE3 C terminus: F1 (amino acids [aa] 475-589), F2 (aa 590-667), F3 (aa 668-747), and F4 (aa 748-832) (Cha *et al.*, 2006). His₆-tagged fusion proteins were expressed in *Escherichia coli* Rosetta 2 (DHE-3) cells, and crude cell extract was prepared following the Invitrogen protocol under native conditions. Proteins were affinity-purified with Ni²⁺-nitrilotriacetic acid resin as suggested by the manufacturer (QIAGEN, Valencia, CA).

In Vitro Phosphorylation Assay by CK2 α

In vitro phosphorylation experiments were carried out by incubating purified GST-CK2 α fusion protein with synthetic peptide (100 μM) containing either S⁷¹⁹ or A⁷¹⁹ for 10 min at 30°C in a solution consisting of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 μM EGTA, and 25 μM [γ -³²P]ATP (final volume 50 μl). Two synthetic peptides (mimicking rabbit NHE3), 1) S⁷¹⁹ (wild-type), KERLELESDPEEAPD and 2) S⁷¹⁹A, KERLELEADPEEAPD were used in this experiment. Then, 40 μl of each reaction was spotted onto the center of a 2.5-cm P81 phosphocellulose paper, and reaction was terminated by immediately immersing the paper into 75 mM phosphoric acid. Phosphocellulose papers were washed three times in 75 mM phosphoric acid, rinsed briefly with acetone, and air-dried. Radioactivity was measured in a liquid scintillation counter. In this assay, kinase reactions without substrate or without CK2 α were considered as background or blank.

Coimmunoprecipitation (IP) of NHE3 with CK2

IP was performed using lysates from PS120/NHE3 or PS120/NHE3-S719A cells, epitope tagged with HA, by using VSG-G as a negative control. Cells were grown in 10-cm dishes at ~100% confluence and serum starved for 3 h; cell lysate was prepared in lysis buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM EDTA trisodium, 3 mM EGTA, 1 mM Na₃VO₄, and 1% Triton X-100 with protease inhibitor cocktail [Sigma-Aldrich]). Aliquots (1.5 mg of protein in 1 ml) of lysate were incubated overnight with 30 μl of either monoclonal anti-HA affinity agarose (Roche Diagnostics, Indianapolis, IN) or monoclonal anti-VSV-G antibodies conjugated to agarose beads at 4°C in a rotator (antibody conjugated agarose beads were prewashed with lysis buffer [as described above] before use). The antibody conjugated protein A-Sepharose beads were gently spun down and washed five times with lysis buffer. Bound proteins were eluted with 2 $\times$ sample buffer, and proteins were separated by SDS-PAGE and transferred to nitrocellulose. The blots were probed with polyclonal anti-CK2 α or anti-CK2 β (Abcam) or monoclonal anti-HA primary antibody (Covance Research Products), followed by fluorescently labeled secondary antibody (IRDye 800 or 680) for visualization according to the manufacturer's protocol. Protein bands were visualized by the Odyssey system (LI-COR, Lincoln, NE).

In Vitro Binding Assays

Far Western Blot Analysis. An overlay approach was used to examine the direct interaction of purified recombinant His-tagged F1, F2, F3, and F4 fragments of NHE3 C terminus on blots with a purified GST-fusion protein encoding full-length CK2 α . Identification was with CK2 α antibody. Recombinant F1, F2, F3, and F4 (3 μg) were separated by 14% SDS-PAGE, transferred to nitrocellulose, and blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) (10 mM potassium phosphate buffer, pH 7.4, and 0.15 M NaCl) for 1 h. The blots were incubated with 5 ml of GST-CK2 α (5 $\mu\text{g}/\text{ml}$) in 5% milk/PBS for 16–20 h at 4°C and then gently washed with 0.05% Tween/PBS three times for 5 min each. The blots were incubated with polyclonal CK2 α antibody (1:1000) for 1 h, washed with 0.05% Tween/PBS three times for 5 min each, and incubated with fluorescently labeled, IRDye 800-conjugated goat anti-rabbit secondary antibody (Rockland Immunochemicals, Gilbertsville, PA) for 1 h. Finally, they were washed three times for 5 min each with 0.05% Tween/PBS and one time with PBS. Protein bands were detected by Odyssey system (LI-COR).

Pull-Down Assays. CK2 α -GST fusion proteins were purified using glutathione-Sepharose beads as described above and left on the beads for pull-down assays. Pull-down assays were performed by incubating 5 or 10 μl of CK2 α -GST beads with purified recombinant proteins, F1, F2, F3, and F4 fragments of C-terminal NHE3 (5 $\mu\text{g}/\text{ml}$) in binding buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40, 200 μM Na₃VO₄, and protease

inhibitors) overnight at 4°C. Complexes were pelleted at 10,000 $\times g$ for 2 min and washed three times in 1 ml of lysis buffer. Bound proteins were eluted from the beads by heating in sample buffer for 5 min at 80°C. Proteins were separated in 14% SDS-PAGE, transferred to nitrocellulose membranes, and visualized by Ponceau S staining.

Cell Surface Biotinylation

NHS-SS-biotin was used to determine the percentage of total NHE3 on the plasma membrane; rates of exocytosis and endocytosis; and with ³⁵S labeling, delivery rates of newly synthesized NHE3 to the plasma membrane, as described previously (Cha *et al.*, 2006). PS120 cells stably expressing HA-NHE3-WT and HA-NHE3-S719A were grown to 70–80% confluence in 10-cm Petri dishes. The cells were then serum starved for ~4 h. Cells were rinsed three times with ice-cold phosphate-buffered saline (150 mM NaCl and 20 mM Na₂HPO₄, pH 7.4) and once in borate buffer (154 mM NaCl, 1.0 mM boric acid, 7.2 mM KCl, and 1.8 mM CaCl₂, pH 9.0). For surface labeling of NHE3, cells were incubated with 0.5 mg/ml NHS-SS-biotin (biotinylation solution; Pierce Chemical) for 20 min and repeated once. After labeling, cells were washed three times with the quenching buffer (20 mM Tris and 120 mM NaCl, pH 7.4) to scavenge the unreacted biotin. Cells were washed three times with ice-cold phosphate-buffered saline and solubilized with 0.8 ml N⁺ buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM Na₃EDTA, 3 mM EGTA, and 1% Triton X-100). Lysate, representing the total postnuclear fractions, was incubated with streptavidin-agarose beads for 16 h (overnight). After avidin-agarose precipitation, the supernatant was retained as the intracellular fraction. The avidin-agarose beads were washed five times in N⁺ buffer to remove nonspecifically bound proteins. The avidin-agarose bead bound proteins, representing plasma membrane NHE3, were solubilized in equivalent volumes of loading buffer (5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 1% 2-mercaptoethanol), boiled for 5 min, size-fractionated by SDS-PAGE (10% gels), and then electrophoretically transferred to nitrocellulose. After blocking with 5% nonfat milk, the blots were probed with a monoclonal anti-HA antibody. Western analysis and the quantification of the surface fraction were performed using the Odyssey system and Odyssey software (LI-COR). Multiple volumes for each total, surface, cytosol sample were used with linear regression with intensity of signal to obtain a single value for each sample. Percentage of surface NHE3 was calculated from the ratio [(surface NHE3 signal/total NHE3 signal) $\times$ dilution factor of surface and total NHE3 samples] and expressed as percentage of total NHE3.

Metabolic Pulse-Chase Labeling

To measure the newly synthesized NHE3 that was delivered to the plasma membrane, PS120/NHE3-WT and NHE3-S719A cells in Met/Cys-free DMEM medium for 1 h at 37°C were pulse labeled in the same medium containing Tran³⁵S-Label reagent (Met/Cys, 0.3 mCi/ml; Vantage Pharmaceuticals, Costa Mesa, CA) for 30 min. The extracellular pulse media were quickly removed by washing with cold DMEM. Cells were then chased for 0, 30, 60, 120, and 180 min at 37°C in PS120 media containing 10 $\times$ nonradiolabeled Cys and Met. At each time point, cells were cooled to 4°C, and cell surface biotinylation was performed as described above. Subsequently, the cells were lysed, and biotinylated surface proteins were isolated by streptavidin beads and IP with anti HA-antibody. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose. Detection of ³⁵S-labeled NHE3 and NHE3-S719A mutant were after 24–48 h by using the Storm 860 PhosphorImager system (GE Healthcare). Intensity measurements of ³⁵S-labeled NHE3-WT and NHE3-S719A mutant were quantified with MetaMorph software (Molecular Devices, Sunnyvale, CA). Western analysis was then performed on the same samples using anti-HA antibody by the Odyssey system.

Exocytic Insertion of NHE3 into the Plasma Membrane

To measure exocytic insertion of NHE3 (also called endocytic recycling), N-hydroxysuccinimide (NHS)-reactive sites on proteins on the PS120 cell surface were first blocked by pretreatment with sulfo-NHS-acetate with some slight modifications (Cha *et al.*, 2006). Cells were rinsed twice with PBS-Ca-Mg (PBS with 0.1 mM Ca²⁺ and 1 mM Mg²⁺) at 4°C. The apical surface was then exposed to 1.5 mg/ml sulfo-NHS-acetate in 0.1 M sodium phosphate, pH 7.5, and 0.15 M NaCl (3 times 40 min at 4°C) to saturate NHS-reactive sites on the cell surface. After quenching (PBS containing 1 mM MgCl₂, 0.1 mM CaCl₂, and 100 mM glycine) for 20 min at 4°C, the cells were rinsed with PBS and then incubated in serum-free normal PS120 media at 37°C for 10 min to allow intracellular NHE3 to reach the plasma membrane (exocytosis). Cells were then treated with 0.5 mg/ml sulfo-NHS-SS-biotin as described above and lysed with N⁺ buffer. The biotinylated fraction, which represents newly inserted surface proteins, was precipitated by streptavidin-agarose, and the precipitate was subjected to SDS-PAGE and Western blotting with anti-HA antibody, as described above.

Endocytosis of NHE3

Endocytosis was measured by a protocol described previously (Cha *et al.*, 2006). PS120 cells expressing NHE3-WT and NHE3-S719A mutant were surface-labeled with sulfo-NHS-SS-biotin at 4°C. Cells were then warmed to 37°C

Figure 1. A novel phosphorylation site at NHE3-S⁷¹⁹ was identified by nanoLC-MS/MS analysis of immunopurified NHE3. (A) Approximately 2 μ g of NHE3 was purified by IP using anti-VSV-G monoclonal antibody from PS120/NHE3-VSV-G cells. Note duplicate lanes of purified NHE3 were separated by SDS-PAGE and stained with Coomassie Blue. The amount of NHE3 was estimated by comparison with amounts of BSA loaded on the same gel. (B) The purified NHE3 was confirmed by Western blot analysis using anti-VSV-G antibody. 1% of the total purified NHE3 was used in the analysis (lane 2). Anti-FLAG antibody was used as a negative control for IP (lane 1). (C) Sequence coverage of NHE3 by nanoLC-MS/MS analysis: 50% coverage of the entire C terminus (underlined) was achieved. The two phosphorylation sites found were S⁵⁵⁴ and S⁷¹⁹, which are shown in a black square/oval, respectively. Peptides identified (16) by mass spectroscopy are in bold, with overlapping peptides separated by a vertical line. (D) Mass spectroscopy: MS/MS analysis of the identified two peptides, one with a doubly charged phosphopeptide identified the phosphorylation site at S⁵⁵⁴ (top) and the other with a triple charged phosphopeptide identified the S⁷¹⁹ phosphorylation site (bottom).

for 0, 5, 10, 15, and 30 min, respectively, and rinsed with cold phosphate-buffered saline twice at 4°C. Surface biotin was cleaved by rocking cells with 150 mM reduced glutathione (GSH) containing buffer at 4°C for 30 min. The biotin bound to freshly endocytosed proteins is protected from the GSH cleavage. Cells were solubilized in N⁺ buffer, and biotinylated proteins were retrieved with streptavidin-agarose affinity precipitation, and internalized and total cellular NHE3 was detected by Western analysis. Intensities of each band were quantitated using Odyssey software. Endocytosis was normalized to surface NHE3 or NHE3-719S present initially.

RESULTS

Identification of a Unique NHE3 Phosphorylation Site

NHE3 under basal conditions is phosphorylated. To identify the phosphorylation sites in NHE3, VSV-G-tagged rabbit NHE3 (E3V) expressed in PS120 cells was immunoprecipitated by monoclonal antibodies against the VSV-G epitope

and resolved by 1D SDS-PAGE. The immunoprecipitated NHE3 was visualized by Coomassie Blue stain (Figure 1A) and further confirmed by SDS-PAGE and Western blot with anti-VSV-G antibody (Figure 1B). NHE3 was excised from the gel and digested by trypsin. The generated tryptic peptides were then analyzed by liquid chromatography tandem mass spectrometry (nanoLC-MS/MS), which identified a total of 13 unique peptides corresponding to ~30% of the protein sequence (Figure 1C). As shown in Figure 1D, two of the identified peptides, one doubly charged (m/z 500.28) and one triply charged peptide (m/z 636.33), showed a characteristic loss of phosphoric acid (loss of 98 Da), which corresponds to the conversion of either phosphoserine or phosphothreonine into dehydroalanine or dehydroaminobutyric acid, respectively. Analysis of the doubly charged phos-

phopeptide identified the specific phosphorylation site to be S⁵⁵⁴ (552RGpSLAFIR₅₅₉; Figure 1D, top), which is a known protein kinase A (PKA) phosphorylation site. Complete y- and b-ion series were identified, both confirming S⁵⁵⁴ as being phosphorylated by the conversion of phosphoserine into dehydroalanine. MS/MS analysis of the triply charged phosphopeptide identified S⁷¹⁹ (715ELELPDPEEAPDYEEAEK₇₃₂) as a unique phosphorylation site in NHE3, not previously identified (Figure 1D, bottom). Phosphorylation of S⁷¹⁹ was also confirmed by a series of y- and b-ion series as described above. We focused further attention on this newly identified S⁷¹⁹ phosphorylation site.

In Vitro Phosphorylation of NHE3 S⁷¹⁹ by Recombinant CK2 α

The ScanSite bioinformatics program (Pfam based) was used to examine the NHE3 protein sequence for kinase phosphorylation sequences (Obenauer *et al.*, 2003) and identified S⁷¹⁹

Figure 2. Recombinant protein kinase CK2 α phosphorylates S719 of NHE3 in vitro. (A) The newly identified S⁷¹⁹ phosphorylation site is conserved across mammalian species in both NHE3 and NHE5 (the NHE isoform that is most related to NHE3). Please note that the S⁷¹⁹ is surrounded on both N- and C-terminal sides by negatively charged amino acids. *, identity; :, homologous residues; ., nonconserved residues. (B) An in vitro kinase reaction was carried out at 30°C with [³²P- γ]ATP containing purified recombinant CK2 α at several amounts (375, 75, and 7.5 ng of protein) and 100 μ M synthetic peptides containing S⁷¹⁹ or A⁷¹⁹ of NHE3. After 10 min, each reaction done in duplicate was terminated. Average radioactivity counts of two separate experiments are shown.

as a substrate for CK2 phosphorylation. The consensus sequence for CK2 is (S/T)XX(D/E). NHE3 aa 719-722 conforms to this sequence and is conserved across species in NHE3; it also occurs in the related NHE5 (Figure 2A). An in vitro kinase assay was used to confirm that CK2 could phosphorylate this sequence in NHE3. A synthetic peptide of rabbit NHE3 aa 712-726 including S⁷¹⁹ and a control peptide, identical except with S719A, were prepared (Figure 2B), and an in vitro kinase assay was performed with recombinant CK2 α . As shown in Figure 2B, a significant amount of ³²P was incorporated when recombinant GST-CK2 α was incubated with NHE3-S⁷¹⁹ peptide but not with the NHE3-S719A peptide or if no substrate was used. Also, when CK2 α was diluted 50-fold, no incorporation of ³²P occurred. These results show that the α subunit of CK2 (catalytic subunit) can directly phosphorylate NHE3-S⁷¹⁹.

Phosphorylation of NHE3 by CK2 Is Required for Basal NHE3 Activity

To assess the effect of phosphorylation at S⁷¹⁹ on NHE3 activity, S⁷¹⁹ was mutated to Ala and stable PS120 cell lines were generated with triple HA-tag NHE3-WT (wild type) and NHE3-S719A mutant. Na⁺/H⁺ exchange rates were determined over a range of intracellular pH_i values by measuring Na⁺-induced recovery from an acid load. The calculated average maximum activity, V_{max} of NHE3-WT was 2092 $\pm$ 55 μ M/s, from four independent experiments in triplicate (Figure 3), whereas cells with mutation of NHE3-S719A resulted in reduced Na⁺/H⁺ exchange rates (V_{max} of 642 $\pm$ 39 μ M/s, from three independent experiments in triplicate). In addition, NHE3-S719A had an increased K'_i(H⁺), 0.14 $\pm$ 0.01 mM versus 0.24 $\pm$ 0.01 mM, p < 0.01. Thus, S⁷¹⁹ is necessary for NHE3 activity under basal conditions. In contrast, NHE3-S719D was not significantly different from wild-type NHE3 (V_{max} of 1827 $\pm$ 74 μ M/s, K'_i(H⁺), 0.15 $\pm$ 0.01 mM).

Figure 3. S719A but not S719D mutation significantly reduced NHE3 activity in PS120 cells. Basal transport activity of NHE3-WT and NHE3-S719A and NHE3-S719D mutants were measured in stably transfected PS120 cells. Cells were loaded with BCECF-AM dye in Na⁺/NH₄Cl medium before being mounted in a spectrofluorometer cuvette. NHE3 activity was then assessed as the rate of Na⁺-dependent cell pH recovery from an acid load. Results shown are from three to four experiments. Kinetic parameters from three to four experiments are shown as mean $\pm$ SEM.

Figure 4. Na^+/H^+ exchange activities of NHE3-WT is sensitive to CK2-specific inhibitors but NHE3-S719A mutant is resistant to DMAT inhibition. PS120/HA-NHE3-WT and HA-NHE3-S719A cells were grown on glass coverslips at 60–80% confluence. Cells on the coverslips were simultaneously loaded with BCECF dye and

NHE3 Wild-Type Activity Is Inhibited by CK2 Inhibitors, whereas the S719A Mutant Has Reduced Basal Activity, Which Is Resistant to CK2 Inhibitors

Several specific inhibitors of CK2 are available commercially. We hypothesized that if CK2 were involved in basal regulation of NHE3, then 1) pharmacologic inhibition of CK2 activity would decrease NHE3 activity and 2) NHE3-S719A mutant would be at least partially resistant to CK2 inhibitors. To investigate this hypothesis, we measured Na^+/H^+ exchange activity in PS120/HA-NHE3-WT and HA-NHE3-S719A cells in the absence and presence of CK2 inhibitors TBB and DMAT (Ruzzene *et al.*, 2002; Sarno *et al.*, 2002; Pagano *et al.*, 2004). TBB and DMAT both caused concentration-dependent inhibition of NHE3 activity with different effects in NHE-WT and the NHE3-S719A mutant. DMAT, a specific inhibitor of CK2 (Obenauer *et al.*, 2003; Cha *et al.*, 2006), inhibited NHE3 wild-type activity by 55% at 30 μM (Figure 4A). TBB, a potent inhibitor of both CK2 and CK1 (it inhibits CK1 at higher concentrations) (Sarno *et al.*, 2002; Ruzzene *et al.*, 2002) exerted a maximum of 85% inhibition of wild-type NHE3 activity at 30 μM (similar effect at 100 μM) and caused 50% inhibition at 3–5 μM TBB (Figure 4B). For both DMAT and TBB, effects were both on $V_{\max}$ (shown in Figure 4, A and B) and increased $K'(\text{H}^+)_i$ (data not shown).

In contrast to these results, NHE3-S719A mutant was resistant to DMAT, with no significant effect at 30 μM (Figure 4A). Similar lack of effect on NHE3-S719A was observed at 5 μM TBB (5 μM TBB inhibits only CK2 and caused ~50% inhibition of NHE3-WT activity). We conclude that S719 is the single site in NHE3 which is phosphorylated by CK2 and is involved in setting basal NHE3 activity. Also, because 30 μM (Figure 4B) and 100 μM (data not shown) TBB caused ~60% inhibition of NHE3-S719A suggests a role for CK1 (or another kinase inhibited by TBB) in setting basal NHE3 activity.

Inhibition of NHE3 activity was also demonstrated in an intestinal epithelial cell model, Caco-2/BBE. In Caco-2/HA-NHE3-WT cells, both TBB and DMAT inhibited NHE3 activity (Figure 4C). However, 30 μM TBB had a significantly larger inhibitory effect on NHE3 compared with DMAT (when both effects were expressed as percentage of inhibition of basal NHE3 activity), also suggesting a role for both CK2 and CK1 (inhibited by high concentration of TBB but not DMAT) in NHE3 regulation in Caco-2 cells.

CK2 α Associates with NHE3

CK2 α has been shown to bind to interacting proteins in addition to phosphorylating them (Shi *et al.*, 2002; Bildl *et al.*, 2004; Treharne *et al.*, 2007). NHE3 is regulated as part of multiprotein complexes. To determine whether CK2 associ-

treated (except control) with CK2 inhibitors DMAT (A) or TBB (B) (at indicated concentrations, 0–30 μM) in $\text{Na}^+/\text{NH}_4\text{Cl}$ medium for 20 min before being mounted in a spectrofluorometer cuvette. TBB and DMAT were also present in TMA and Na^+ solutions. Na^+/H^+ exchange rates (H^+ efflux) are shown from a single experiment (in triplicate), which was repeated three times with similar results. (C) Similar studies on NHE3 activity were performed with TBB and DMAT (30 μM) in Caco-2/HA-NHE3 cells. Results shown are means $\pm$ SEM of initial rates, with results expressed as percentage of each experiment's untreated controls. N is number of experiments. P values are comparison of percentage of inhibition by DMAT and TBB.

Figure 5. NHE3 physically associates with CK2. (A) NHE3-WT Coimmunoprecipitates CK2α. PS120/HA-NHE3-WT cells were grown in 10-cm dishes and serum starved for 3 h. Then, 1.5 mg of total cell lysate was mixed with anti-HA agarose or anti-VSV-G agarose (negative control) overnight at 4°C in a rotator. After five gentle washes, proteins were eluted by adding 50 μ l of SDS loading buffer. Proteins were separated by SDS-PAGE, blotted to nitrocellulose membrane, and probed with anti-HA, anti-CK2α, and anti-CK2β antibodies, as indicated. A negative control was PS120/HA-NHE3 IP with anti-VSVG antibodies. Post-IP refers to supernatant after removal of IP. (B) NHE3-S719A coimmunoprecipitates CK2α in PS120/HA-NHE3-S719A cells. Cells were grown in 10-cm dishes and serum starved for 3 h. Cell lysate was prepared in lysis buffer including 1 mM Na_3VO_4 and 1% Triton X-100 with protease inhibitor cocktail. Then, 1.5 mg of total cell lysate was mixed with anti-HA agarose overnight at 4°C in a rotator. After five gentle washes, proteins were eluted by adding 50 μ l of SDS loading buffer. Proteins were separated by SDS-PAGE, blotted to nitrocellulose membrane and probed with anti-HA, anti-CK2α, and anti-CK2α antibodies, as indicated. PS120/NHE3-VSVG (not HA-tagged) cells were studied on parallel as a negative control.

ated with NHE3 as part of basal function, coimmunoprecipitation was performed using PS120/HA-NHE3 cells (Figure 5A). Cell lysate prepared from PS120 cells stably expressing HA-tagged NHE3 was incubated with anti-HA conjugated to agarose beads. As a negative control, equal volume of the same cell lysate was incubated with anti-VSV-G agarose (NHE3 was not VSV-G epitope-tagged in these studies). As shown in Figure 5A, CK2α but not CK2β coimmunoprecipitated with NHE3. The relationship between NHE3 and CK2α was further examined, asking whether the CK2 phosphorylation of NHE3 at S719 affected their coprecipitation. HA-NHE3 was IP from total lysates of PS120/HA-NHE3-WT and HA-NHE3-S719A cells, respectively. As shown in Figure 5B, CK2 was coimmunoprecipitated equally with NHE3-WT and the NHE3-S719A mutant, indicating that CK2 phosphorylation of NHE3 is not required for the binding of CK2 with NHE3. This also suggests that the binding of CK2 occurs at sites other than at which NHE3 is phosphorylated.

CK2α Directly Binds the NHE3 C Terminus In Vitro

Coimmunoprecipitation does not imply direct binding, because interactions may exist that involve multiple proteins.

Figure 6. Binding of CK2α with C-terminal region of NHE3 at aa 590-667. (A) Pull-down by CK2α recombinant protein of different fragments of NHE3 C terminus (fragments F1–4). Five or 10 μ l of glutathione-Sepharose beads with GST-CK2α attached (top) were incubated overnight with 3 μ g/ml His₆-tagged purified F1 (aa 475-589), F2 (aa 590-667), F3 (668-747), and F4 (748-832) proteins separately. After washing, proteins were eluted and separated with SDS-PAGE (14% gel) and transferred to nitrocellulose membranes. Protein bands were visualized by Ponceau S staining. Only the F2 fragment was detected in the pull-down by Ponceau S shown in the bottom panel. (B) Far Western blot (overlay assays) showed direct binding of NHE3-F2 domain with CK2α. Purified GST-CK2α fusion protein (5 μ g/ml) was incubated with an immunoblot that contained His₆-F1, F2, F3, and F4 fragments of NHE3 C terminus (3 μ g of each), and they were separated by SDS-PAGE and transferred to nitrocellulose. Input of the four fragments transferred to nitrocellulose membrane were detected by Ponceau S is shown in the top panel. Binding of CK2 was only to the F2 fragment as detected by immunoblot with anti-CK2α antibody (bottom).

To determine whether NHE3 and CK2α directly interact and whether the sites of interaction and phosphorylation are different, an in vitro binding assay was performed by incubating recombinant GST-CK2α (~10 μ g) bound to glutathione-Sepharose beads with different purified NHE3 C-terminal fusion H₆-proteins F1 (aa 475-589), F2 (aa 590-667), F3 (aa 668-747), and F4 (aa 748-832), respectively. Two different amounts of purified GST-CK2α were used for binding and are shown in Figure 6A, top. The fragment of NHE3 C terminus bound to CK2α was visualized by Ponceau S staining (Figure 6A). Binding only to the F2 fragment (aa 590-667) was observed when membrane was immunoblotted with anti-H6 antibody. From this binding assay, we concluded that CK2α specifically binds to aa 590-667 of the NHE3 C terminus. This binding site is upstream of the CK2 NHE3-S719 phosphorylation site. A similar conclusion came from an in vitro Far Western blot analysis (Figure 6B).

Mutation of the CK2 Phosphorylation Site NHE3-S719A Decreases the Surface Amount of NHE3

To determine the basis of the reduced Na^+/H^+ exchange activity of NHE3-S719A, the amount of protein expressed and plasma membrane expression were determined using immunoblot (IB) and cell surface biotinylation studies. In PS120 cells stably expressing HA-NHE3-WT and HA-NHE3-S719A, the total amount of NHE3 protein and surface-expressed protein were compared by using immuno-

Figure 7. Amount of plasma membrane NHE3-S719A is reduced as determined by surface biotinylation assay. PS120 cells stably expressing HA-NHE3-WT and HA-NHE3-S719A were grown in 10-cm dishes and biotinylated with NHS-SS-biotin. Cells were then lysed in equal volumes of HEPES buffer containing 1% Triton X-100 at 4°C. Of the 0.8 ml of cell lysate, 0.72 ml was incubated with streptavidin-agarose beads to isolate the biotinylated proteins. Amount of NHE3 present in the biotinylated fractions was considered as surface NHE3. Biotinylated protein was eluted from the beads with 80 μl of sample buffer. The surface protein samples were 9 times concentrated compared with the total cell lysate sample. Multiple dilutions of total, surface and nonavidin precipitated (referred to as cytosolic) fractions were loaded (volume) in SDS-gels. NHE3 protein abundance was quantified by immunoblot with anti-HA antibody (A) by using anti-GAPDH as a loading control. The intensity of each band was quantitated using Odyssey software and the amount of NHE3 calculated in each fraction using linear regression of the different sample volumes and intensity. Bar graphs show the calculated mean $\pm$ SEM amount of surface NHE3 as percentage of total NHE3 (described in *Materials and Methods*) from similar experiments (B). NHE3-S719A mutant had ~50% less surface NHE3 compared with wild-type ($p < 0.01$).

blots of total cell lysate and surface biotinylated protein. As shown in Figure 7, total NHE3 expression was similar in NHE3-WT and NHE3-S719A. In contrast, surface expression of NHE3-S719A was ~50% of NHE3-WT expression in PS120 cells (Figure 7, A and B). These results indicate that mutation of the CK2 phosphorylation site at NHE3-S719 has no effect on total cellular expression of NHE3 but reduced the plasma membrane amount of NHE3 (~50%), which was consistent with the reduced Na^+/H^+ activity of the NHE3-S719A mutant.

The CK2 Inhibitor DMAT Reduces the Surface Expression of NHE3 in PS120 Cells

Because NHE3-S719A mutation caused lower expression of NHE3 on the cell surface, we speculated that the CK2 specific inhibitor, DMAT treatment might also lower the surface expression of NHE3. PS120 cells expressing NHE3-WT were treated with either DMAT (100 μM) or dimethyl sulfoxide (DMSO) for 30 min. Treated cells were biotinylated at 4°C, and total and surface amount of NHE3 were detected by Western analysis and quantitated. As shown in Figure 8, DMAT-treated cells have ~40% lower plasma membrane

Figure 8. CK2 inhibitor DMAT reduces surface expression of NHE3 in PS120 cells. PS120 cells expressing NHE3 were grown in 10-cm dishes, cells were serum starved for 2 h and then treated with DMSO (control) or 100 μM DMAT for 30 min at 37°C, before biotinylation as in Figure 7. Detection and quantitation of total and surface amount of NHE3 with DMSO and DMAT treatments are shown in A. Surface expression of NHE3 was determined as a percent of total NHE3. Results are mean $\pm$ SEM from three experiments. (B) DMAT treated cells had ~40% less surface NHE3 expression than DMSO-treated cells. The p value is comparison of surface NHE3 of wild-type NHE3 exposed to DMAT versus just DMSO ($p < 0.01$).

expression of NHE3 compared with DMSO-treated cells. This result further supports that CK2 is involved in NHE3 trafficking to the cell surface.

CK2 Regulates Trafficking of NHE3 to the Plasma Membrane

That the NHE3-S719A mutant and DMAT treatment of NHE3-WT reduced NHE3 surface expression without a change in total cellular expression further indicates that CK2 phosphorylation of S⁷¹⁹ might play a role in NHE3 trafficking. Mechanistic studies were undertaken to define the role of CK2 phosphorylation of NHE3 in increasing surface expression. We performed pulse-chase experiments in the PS120 cells, metabolically labeled with [³⁵S]Met/Cys and compared the delivery of newly synthesized NHE3 to the cell surface with various times of chase in NHE3-WT and S719A mutant cells. As shown in Figure 9, A–C, cells expressing NHE3-S719A had a reduced rate of delivery of newly synthesized NHE3 (³⁵S labeled newly synthesized NHE3) to the cell surface compared with the NHE3-WT. There was an increasing amount of NHE3-WT for 60 min and of NHE3-S719A for 90 min (Figure 9, B and C). After peak amounts of newly synthesized NHE3-WT/NHE3-S719A were reached on the surface, both decreased over ~90 min, which is compatible with similar membrane retention of NHE3-WT and NHE3-S719A (Figure 9, B and C). This result showed that CK2 phosphorylation of S719A was nec-

Figure 9. Delivery of newly synthesized NHE3 is decreased in NHE3-S719A mutant, whereas plasma membrane retention is normal. (A–C) Metabolic pulse-chase/surface biotinylation experiments were performed to determine the surface delivery of newly synthesized NHE3. PS120 cells expressing HA-NHE3-WT and HA-NHE3-S719A were starved of Met/Cys for 60 min and then pulse labeled with 0.3 mCi/ml [35 S]Met/Cys for 30 min at 37°C. The cells were then chased in normal medium supplemented with unlabeled Cys and Met at 37°C for 0, 30, 60, 120, 150, and 180 min. After completion of each time, cells were chilled to 4°C, surface biotinylated, and lysates were precipitated with streptavidin and separated by SDS-PAGE. Then, biotinylated surface [35 S]NHE3-WT and NHE3-S719A were detected by autoradiography and immunoblotted using HA-antibody (A). The intensity of 35 S surface NHE3-WT and NHE3-S719A were quantitated by MetaMorph, normalized to amount of surface NHE3, and plotted versus time. (B) A single experiment. (C) Mean $\pm$ SEM of four experiments. Rate of delivery of newly synthesized NHE3-S719A mutant was decreased compared with wild-type NHE3. Wild-type NHE3 and NHE3-S719A both had similar retention after peak delivery of newly synthesized NHE3 (see C). All points in C except 0 were significantly different between wild-type NHE3 and NHE3-S719A (p at least <0.05).

essary for delivery of newly synthesized NHE3 to the plasma membrane but not for its retention once there.

Because the surface amount of NHE3 is also dependent on the balance of exocytic insertion and endocytic internalization, we further examined whether exocytic insertion to the cell surface and endocytosis of NHE3 from the cell surface were affected by the NHE3-S719A mutation. As shown in Figure 10A, exocytic insertion of NHE3 in the cell surface of the NHE3-S719A mutant was lower compared with NHE3-WT cells. These results plus those in Figure 9 show that the trafficking to the plasma membrane of NHE3 via both the synthetic pathway and from endocytic recycling were reduced in the absence of CK2 phosphorylation of NHE3. In contrast, shown in Figure 10, B and C, endocytosis of NHE3, which also was determined using cell surface biotinylation, was not affected by mutation of NHE3-S719A (measured over 15 min).

DISCUSSION

NHE3 is known to be partially active under basal conditions (Donowitz *et al.*, 1985; Donowitz and Welch, 1986; Levine *et al.*, 1995). This characteristic of NHE3 has been identified in every cell system in which it has been studied, in intact intestine, in polarized epithelial cell models, and in fibroblasts. However, the molecular mechanism that maintains this partial activation has not been identified. This study identified that CK2 stimulates NHE3 activity under basal conditions. CK2 binds to the NHE3 C terminus and stimu-

lates NHE3 activity by phosphorylating the NHE3 C terminus at a single site (S⁷¹⁹) that is different from the CK2 binding site. This is an example of the NHE3 C terminus acting as a scaffold for proteins that regulate it. Being set at a partially activated state allows NHE3 to be both inhibited, as occurs during the early phases of digestion, and further stimulated, as occurs in the later stages of digestion. This is in contrast to other transporters which are also regulated by trafficking, such as GLUT4, which are almost entirely intracellular under basal conditions and which traffic to the surface with stimuli, such as insulin, which leads to much greater-fold stimulation of both activity and percentage of increase in amount in the plasma membrane, compared with NHE3 (Pessin *et al.*, 1999; Watson and Pessin, 2001).

CK2 phosphorylation of NHE3 regulates exchanger activity by increasing the percentage of total NHE3 on the plasma membrane by affecting its trafficking. CK2 inhibitors reduced NHE3 activity by $\sim 40\%$ and reduced the percent NHE3 on the plasma membrane by $\sim 50\%$, consistent with the effects being largely by affecting trafficking. The CK2 effects on trafficking consist of increased endocytic recycling (exocytosis) with no effect in rate of endocytosis or total NHE3 expression. In addition, CK2 phosphorylation of NHE3 was necessary for normal delivery of newly synthesized NHE3 to the plasma membrane. Quantitative effects were somewhat different studying the S719A mutant, which reduced NHE3 activity by $\sim 70\%$ while reducing plasma membrane amount NHE3 by $\sim 60\%$. Possibilities for the difference between CK2 drug inhibitors and mutation of S⁷¹⁹

Figure 10. NHE3 exocytosis but not endocytosis is reduced in the NHE3-S719A mutation. (A) Exocytosis: To measure the endocytic recycling (exocytosis), cell surface NHE3-WT or NHE3-S719A were blocked by sulfo-NHS acetate at 4°C for 60 min. Cells were then washed and temperature increased to 37°C in serum-free PS120 medium for 10 min to allow arrival of new NHE3 to the surface. As controls, one set of cells were kept at 4°C throughout, and one set of cells were handled as the others but without sulfo-NHS acetate blocking. NHE3 was identified by IB. Total amount of biotinylated surface NHE3 without sulfo-NHS acetate in cells kept at 4°C is shown in lane 1. Amount of surface NHE3 present after blocking with sulfo-NHS-acetate (0 min) is shown in lane 2. Amount of NHE3-WT and NHE3-S719A at the plasma membrane in 10 min at 37°C is shown in lane 3. After 10 min at 37°C, there was much less newly arrived surface NHE3-S719A than NHE3-WT. (B and C) Endocytosis: PS120/HA-NHE3-WT and PS120/HA-NHE3-S719A cells were exposed to sulfo-NHS-SS-biotin at 4°C for 40 min. After washing out of nonbound biotin, biotinylated cells were warmed to 37°C for indicated times. Then, surface biotin was removed by exposure to reduced glutathione. Internalization of NHE3 from the cell surface was detected as the biotinylated NHE3 protected from the reductive cleavage by reduced-glutathione at 4°C. The internalized biotinylated NHE3 was precipitated with streptavidin beads, separated, and immunoblotted. Samples without reductive cleavage were used as controls to indicate surface NHE3 and to normalize endocytosed NHE3 (lane 1, labeled C top row, in which 50% of sample was loaded on gel). Second row (lane 1, labeled C) was total NHE3 (6% of total sample was loaded on gel). (C) Internalized

include that S719A affects NHE3 activity by a mechanism in addition to serving as the CK2 phosphorylation site or that chronic effects of the S719A mutation lead to further secondary changes that do not necessarily directly involve CK2.

The search for unrecognized phosphorylation sites of NHE3 was based on our belief that in spite of NHE3 phosphorylation sites having been identified for cAMP and SGK1, it was likely that there were additional sites present (Donowitz and Li, 2007). This was because, under basal conditions, the S/T phosphatase inhibitor okadaic acid stimulated NHE3 (Levine *et al.*, 1995), implying a role for NHE3 phosphorylation in stimulating NHE3, which cAMP inhibits. In addition to the opposite effects on NHE3 activity of phosphorylation by CK2 versus cAMP, another important difference of the effects of CK2 versus PKA and SGK1, is that the latter are brought into the NHE3 complex by binding NHERF1 (PKAII) and/or NHERF2 (PKAII, SGK1), whereas CK2 is brought into the complex by directly binding NHE3.

A role in NHE3 regulation for CK2 was not surprising. It is a ubiquitous kinase with at least the widely quoted 300 recognized substrates, including all classes of proteins that are found in most cellular compartments (Litchfield, 2003; Olsten and Litchfield, 2004; Meggio and Pinna, 2005). Although nuclear proteins seem to be the most affected (Faust and Montanarh, 2000), CK2 regulates multiple transport proteins in many cell compartments (Table 1).

CK2 is a heterotetramer consisting of two catalytic (α , α^1) and two physically joined regulatory β subunits (Faust and Montanarh, 2000; Filhol *et al.*, 2004). There are examples of

surface NHE3 amount at different times was quantitated using Odyssey software and expressed as a percentage of initial surface amount, after normalization to total amount of NHE3. Zero time internalization was used as background and subtracted from each time point. Results shown are mean $\pm$ SEM of three experiments. Internalization rate of surface NHE3 was similar in NHE3 wild type (open circles) and NHE3-719A mutant cells (closed circles).

both the α and β subunits binding CK2 substrates, with more examples of β (Litchfield, 2003; Olsten and Litchfield, 2004; Bolanos-Garcia *et al.*, 2006). Moreover, the concept that CK2 binds one protein in a complex and regulates function by phosphorylating another protein in the complex seems to be a common mechanism of action of CK2. For example, CK2 binds KSR1 (kinase suppressor of Ras) and phosphorylates Raf in the multiprotein complex of the extracellular signal-regulated kinase (ERK) cascade (Raf, mitogen-activated protein kinase kinase, ERK) (Allen *et al.*, 2007).

Although CK2 generally is thought to function in a constitutive manner, as with the identified NHE3 regulation, there is emerging evidence that it also takes part in regulated functions (Olsten *et al.*, 2005; Theis-Febvre *et al.*, 2005; Allen *et al.*, 2007). Multiple mechanisms have been identified by which CK2 regulates transporter function. In its interactions with the small-conductance Ca^{2+} activated potassium channels, CK2 is in a complex with the channel, calmodulin (CaM), and PP2A (Allen *et al.*, 2007). It regulates channel gating by phosphorylating CaM in the multiprotein complex leading to reduction of the Ca^{2+} sensitivity of the channel (Arrigoni *et al.*, 2004). Cystic fibrosis transmembrane conductance regulator (CFTR) binds and is regulated by CK2 (Treharne *et al.*, 2007). CK2 binds wild-type CFTR at approximately aa 505 (very close to the common mutation Phe Δ 508) and phosphorylates CFTR at S⁵¹¹, which seems necessary for cAMP gating of the channel. Binding of CK2 to CFTR requires the presence of the S⁵¹¹. That is, the substrate phosphorylation site is necessary for nearby CK2 binding. CK2 phosphorylates S¹⁴⁸⁰ of the *N*-methyl-D-aspartate (NMDA) receptor, which leads to reduced binding of this transporter to postsynaptic density 95/disc-large/zona occludens domains of PSD95 and SAP102, as well as reduced NMDA surface expression. Thus, there are examples of CK2 phosphorylating transporters and also binding to and phosphorylating the same part of the protein (CFTR). However, unique aspects of transporter regulation by CK2 demonstrated for NHE3 include regulation of basal transport function, contribution to trafficking with effects on exocytosis and delivery of newly synthesized NHE3 to the plasma membrane, and binding to one site but phosphorylating a separate domain in the protein.

We showed here that CK2 phosphorylation of NHE3-S719 stimulates NHE3 activity. Intriguingly, we demonstrated previously that NHE3 truncated at aa 690 (NHE3-690), in which S⁷¹⁹ was also removed, exhibits a threefold increase in activity due to a threefold increase in the amount of NHE3 at the cell surface. If S⁷¹⁹ were required for the basal stimulation of NHE3, removal of S⁷¹⁹ would have been expected to decrease the basal NHE3 activity. Why did we observe an increase of activity in NHE3-690 instead? We previously identified two endocytosis domains in the C terminus of NHE3: one located at aa 690-756 (which includes S⁷¹⁹) and the other at aa 757-832 (Akhter *et al.*, 2000). Regulation of WT NHE3 is primarily through trafficking, which is dictated by the balance or interplay between exocytosis (via mechanisms such as CK2 mediated S⁷¹⁹ phosphorylation) and endocytosis (via endocytic signals as mentioned above) (Donowitz and Li, 2007). These results indicate that NHE3 aa, which normally stimulate NHE3 activity (S⁷¹⁹), are interspersed with domains that normally inhibit NHE3 (two endocytosis signals) and show that there is intermixing of stimulatory and inhibitory domains in the NHE3 C terminus. How these interspersed signals functionally interact will be important to understand in the future.

In addition to data with mutation of the NHE3 CK2 phosphorylation site S⁷¹⁹, elucidation of the functional role of

CK2 phosphorylation of NHE3 was partially based on study of two pharmacologic inhibitors with high specificity for CK2. DMAT has an IC₅₀ value of 0.14 μM for CK2, with no effect on CK1 at concentrations up to 100 μM (at least 700 $\times$ less sensitive), whereas TBB has an IC₅₀ value of 0.5 μM for CK2 but inhibits CK1, with an IC₅₀ value of 25 μM (50 $\times$ less sensitive) (Sarno *et al.*, 2002, 2005; Ruzzene *et al.*, 2002; Pagano *et al.*, 2004). Although there are several additional putative CK2 phosphorylation sites in the NHE3 C terminus, the fact that there was no significant effect on NHE3-S719 activity of DMAT at up to 100 μM was consistent with there being only a single NHE3 CK2 phosphorylation site that is linked to regulation of basal NHE3 activity. In contrast, the fact that TBB at high concentrations inhibited NHE3-S719A suggests that a kinase in addition to CK2 contributes to basal NHE3 activity. We suggest that, given that CK1 is inhibited by high concentrations of TBB and that the NHE3 C terminus contains several putative CK1 phosphorylation sequences, it is likely that CK1 also contributes to basal NHE3 activity.

This study identified a newly recognized phosphorylation site in the NHE3 C terminus and showed that it was phosphorylated by CK2 and contributed to setting basal NHE3 activity by increasing the amount of NHE3 on the plasma membrane. Moreover, we identified the specific aspects of NHE3 trafficking that were CK2 dependent, which included exocytosis and delivery of newly synthesized NHE3. In contrast, total NHE3 expression, endocytosis and plasma membrane retention were not CK2 dependent. These studies indicate that the NHE3 C terminus not only acts as a scaffold for some of its regulatory proteins but also there seems to be organization of these proteins (in this case, CK2 binding at one site and phosphorylation at another site) along the C terminus to control NHE3 activity.

ACKNOWLEDGMENTS

We acknowledge the expert editorial assistance of H. McCann. This study was supported in part by the National Institute of Health, National Institute of Diabetes and Digestive and Kidney Diseases grants R01-DK26523, R01-DK61765, K01-DK62264, P01-DK072084, and R24-DK64388 (The Hopkins Basic Research Digestive Diseases Development Core Center), and the Hopkins Center for Epithelial Disorders and NIH Roadmap Grant U54RR020839.

REFERENCES

- Akhter, S., Cavet, M. E., Tse, C. M., and Donowitz, M. (2000). C-Terminal domain of Na⁺/H⁺ exchanger isoform 3 are involved in the basal and serum-stimulated membrane trafficking of the exchanger. *Biochemistry* 39, 1990–2000.
- Allen, D., Fakler, B., Maylie, J., and Adelman, J. P. (2007). Organization and regulation of small conductance Ca²⁺-activated K⁺ channel multiprotein complexes. *J. Neurosci.* 27, 2369–2376.
- Arrigoni, G., Marin, O., Pagano, M. A., Settimo, L., Paolin, B., Meggio, F., and Pinna, L. A. (2004). Phosphorylation of calmodulin fragments by protein kinase CK2. Mechanistic aspects and structural consequences. *Biochemistry* 43, 12788–12798.
- Bildl, W. *et al.* (2004). Protein kinase CK2 is coassembled with small conductance Ca(2+)-activated K⁺ channels and regulates channel gating. *Neuron* 43, 847–858.
- Bolanos-Garcia, V. M., Fernandez-Recio, J., Allende, J. E., and Blundell, T. L. (2006). Identifying interaction motifs in CK2 β —a ubiquitous kinase regulatory subunit. *Trends Biochem. Sci.* 31, 654–661.
- Bone, D. B., Robillard, K. R., Stolk, M., and Hammond, J. R. (2007). Differential regulation of mouse equilibrative nucleoside transporter 1 (mENT1) splice variants by protein kinase CK2. *Mol. Membr. Biol.* 24, 294–303.
- Cai, Y. *et al.* (2004). Calcium dependence of polycystin-2 channel activity is modulated by phosphorylation at Ser812. *J. Biol. Chem.* 279, 19987–19995.
- Cha, B., Tse, M., Yun, C., Kovbasnjuk, O., Mohan, S., Hubbard, A., Arpin, M., and Donowitz, M. (2006). The NHE3 juxtamembrane cytoplasmic domain

- directly binds ezrin: dual role in NHE3 trafficking and mobility in the brush border. *Mol. Biol. Cell* 17, 2661–2673.
- Donowitz, M., and Li, X. (2007). Regulatory binding partners and complexes of NHE3. *Physiol. Rev.* 87, 825–872.
- Donowitz, M., and Welsh, M. J. (1986). Ca^{2+} and cAMP in regulation of intestinal Na, K and Cl transport. *Annu. Rev. Physiol.* 48, 135–150.
- Donowitz, M., Wicks, J., Madara, J. L., and Sharp, G. W. (1985). Studies on role of calmodulin in Ca^{2+} regulation of rabbit ileal Na and Cl transport. *Am. J. Physiol.* 248, G726–G740.
- Faust, M., and Montemar, M. (2000). Subcellular localization of protein kinase CK2. A key to its function? *Cell Tissue Res.* 301, 329–340.
- Filhol, O., Martiel, J. L., and Cochet, C. (2004). Protein kinase CK 2, a new view of an old molecular complex. *EMBO Rep.* 5, 351–355.
- Glavy, J. S., Horwitz, S. B., and Orr, G. A. (1997). Identification of the in vivo phosphorylation sites for acidic-directed kinases in murine mdrlb P-glycoprotein. *J. Biol. Chem.* 272, 5909–5914.
- Gronborg, M., Bunkenborg, J., Kristiansen, T. Z., Jensen, O. N., Yeo, C. J., Hruban, R. H., Maitra, A., Goggins, M. G., and Pandey, A. (2004). Comprehensive proteomic analysis of human pancreatic juice. *J. Proteome Res.* 3, 1042–1055.
- Janecki, A. J., Montrose, M. H., Zimniak, P., Zweibaum, A., Tse, C. M., Khurana, S., and Donowitz, M. (1998). Subcellular redistribution is involved in acute regulation of the brush border $\text{Na}^{+}/\text{H}^{+}$ exchanger isoform 3 in human colon adenocarcinoma cell line Caco-2. Protein kinase C-mediated inhibition of the exchanger. *J. Biol. Chem.* 273, 8790–8798.
- Jones, S., and Yakel, J. L. (2003). Casein kinase ii (protein kinase ck2) regulates serotonin 5-HT₃ receptor channel function in ng108–15 cells. *Neuroscience* 119, 629–634.
- Krantz, D. E., Peter, D., Liu, Y., and Edwards, R. H. (1997). Phosphorylation of a vesicular monoamine transporter by casein kinase II. *J. Biol. Chem.* 272, 6752–6759.
- Lamprecht, G., Heil, A., Baisch, S., Lin-Wu, E., Yun, C. C., Kalbacher, H., Gregor, M., and Seidler, U. (2002). The down regulated in adenoma (dra) gene product binds to the second PDZ domain of the NHE3 kinase a regulatory protein (E3KARP), potentially linking intestinal $\text{Cl}^{-}/\text{HCO}_3^{-}$ -exchange to $\text{Na}^{+}/\text{H}^{+}$ exchange. *Biochemistry* 41, 12336–12342.
- Levine, S. A., Montrose, M. H., Tse, C. M., and Donowitz, M. (1993). Kinetics and regulation of three cloned mammalian $\text{Na}^{+}/\text{H}^{+}$ exchangers stably expressed in a fibroblast cell line. *J. Biol. Chem.* 268, 25527–25535.
- Levine, S. A., Nath, S. K., Yun, C. H., Yip, J. W., Montrose, M., Donowitz, M., and Tse, C. M. (1995). Separate C-terminal domains of the epithelial specific brush border $\text{Na}^{+}/\text{H}^{+}$ exchanger isoform NHE3 are involved in stimulation and inhibition by protein kinases/growth factors. *J. Biol. Chem.* 270, 13716–13725.
- Lieberman, D. N. and Mody, I. (1999). Casein kinase-II regulates NMDA channel function in hippocampal neurons. *Nat. Neurosci.* 2, 125–132.
- Litchfield, D. W. (2003). Protein kinase CK 2, structure, regulation and role in cellular decisions of life and death. *Biochem. J.* 369, 1–15.
- Macica, C. M., and Kaczmarek, L. K. (2001). Casein kinase 2 determines the voltage dependence of the Kv3.1 channel in auditory neurons and transfected cells. *J. Neurosci.* 21, 1160–1168.
- Madrid, R., Le Maout, S., Barrault, M. B., Janvier, K., Benichou, S., and Merot, J. (2001). Polarized trafficking and surface expression of the AQP4 water channel are coordinated by serial and regulated interactions with different clathrin-adaptor complexes. *EMBO J.* 20, 7008–7021.
- Meggio, F., and Pinna, L. A. (2005). One-thousand-and-one substrates of protein kinase CK2? *FASEB J.* 17, 349–368.
- Moe, O. W. (1999). Acute regulation of proximal tubule apical membrane $\text{Na}^{+}/\text{H}^{+}$ exchanger NHE-3, role of phosphorylation, protein trafficking, and regulatory factors. *J. Am. Soc. Nephrol.* 10, 2412–2425.
- Murtazina, R., Kovbasnjuk, O., Donowitz, M., and Li, X. (2006). $\text{Na}^{+}/\text{H}^{+}$ exchanger NHE3 activity and trafficking are lipid Raft-dependent. *J. Biol. Chem.* 281, 17845–17855.
- Obenauer, J. C., Cantley, L. C., and Yaffe, M. B. (2003). Scansite 2.0, Proteomic-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res.* 31, 3635–3641.
- Olsten, M. E., and Litchfield, D. W. (2004). Order or chaos? An evaluation of the regulation of protein kinase CK2. *Biochem. Cell Biol.* 82, 681–693.
- Olsten, M. E., Weber, J. E., and Litchfield, D. W. (2005). CK2 interacting proteins: emerging paradigms for CK2 regulation. *Mol. Cell Biochem.* 274, 115–124.
- Pagano, M. A., Meggio, F., Ruzzene, M., Andrzejewska, M., Kazimierczuk, Z., and Pinna, L. A. (2004). 2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole: a novel powerful and selective inhibitor of protein kinase CK2. *Biochem. Biophys. Res. Commun.* 321, 1040–1044.
- Pessin, J. E., Thurmond, D. C., Elmendorf, J. S., Coker, K. J., and Okada, S. (1999). Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! *J. Biol. Chem.* 274, 2593–2596.
- Rosbeek, S. *et al.* (2004). Phosphorylation by protein kinase CK2 modulates the activity of the ATP binding cassette A1 transporter. *J. Biol. Chem.* 279, 37779–37788.
- Ruzzene, M., Penzo, D., and Pinna, L. A. (2002). Protein kinase CK2 inhibitor 4, 5, 6, 7-tetrabromobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (Hs1) in Jurkat cells. *Biochem. J.* 364, 41–47.
- Sarno, S., Moro, S., Meggio, F., Zagotto, G. L., Dal Ben, D., Ghisellini, P., Battistutta, R., Zanotti, G., and Pinna, L. A. (2002). Toward the rational design of protein kinase casein kinase-2 inhibitors. *Pharmacol. Ther.* 93, 159–168.
- Sarno, S., Ruzzene, M., Frascella, P., Pagano, M. A., Meggio, F., Zamboni, A., Mazzorana, M., Di Maira, G., Lucchini, V., and Pinna, L. A. (2005). Development and exploitation of CK2 inhibitors. *Mol. Cell Biochem.* 274, 69–76.
- Shi, H., Asher, C., Yung, Y., Kligman, L., Reuveny, E., Seger, R., and Garty, H. (2002). Casein kinase 2 specifically binds to and phosphorylates the carboxy termini of ENaC subunits. *Eur. J. Biochem.* 269, 4551–4558.
- Theis-Febvre, N., Martel, V., Laudet, B., Souchier, C., Grunwald, D., Cochet, C., and Filhol, O. (2005). Highlighting protein kinase CK2 movement in living cells. *Mol. Cell. Biochem.* 274, 15–22.
- Trehan, K. J. *et al.* (2007). Protein kinase CK2, cystic fibrosis transmembrane conductance regulator, and the deltaF508 mutation: F508 deletion disrupts a kinase-binding site. *J. Biol. Chem.* 282, 10804–10813.
- Wang, D., Sun, H., Lang, F., and Yun, C. C. (2005). Activation of NHE3 by dexamethasone requires phosphorylation of NHE3 at Ser663 by SGK1. *Am. J. Physiol. Cell Physiol.* 289, C802–C810.
- Watson, R. T., and Pessin, J. E. (2001). Intracellular organization of insulin signaling and GLUT4 translocation. *Recent Prog. Horm. Res.* 56, 175–193.
- Wiederkehr, M. R., Zhao, H., and Moe, O. W. (1999). Acute regulation of $\text{Na}^{+}/\text{H}^{+}$ exchanger NHE3 activity by protein kinase C: role of NHE3 phosphorylation. *Am. J. Physiol.* 276, C1205–C1217.
- Yip, K. P., Tse, C. M., McDonough, A. A., and Marsh, D. J. (1998). Redistribution of $\text{Na}^{+}/\text{H}^{+}$ exchanger isoform NHE3 in proximal tubules induced by acute and chronic hypertension. *Am. J. Physiol.* 275, F565–F575.
- Zachos, N. C., Tse, M., and Donowitz, M. (2005). Molecular physiology of intestinal $\text{Na}^{+}/\text{H}^{+}$ exchange. *Annu. Rev. Physiol.* 67, 411–443.