The Sodium-Hydrogen Exchanger NHE1 Is an Akt Substrate Necessary for Actin Filament Reorganization by Growth Factors\textsuperscript{a}\textsuperscript{b}\textsuperscript{c}

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The kinase Akt mediates signals from growth factor receptors for increased cell proliferation, survival, and migration, which contribute to the positive effects of Akt in cancer progression. Substrates are generally inhibited when phosphorylated by Akt; however, we show phosphorylation of the plasma membrane sodium-hydrogen exchanger NHE1 by Akt increases exchanger activity (H\textsuperscript{+} efflux). Our data fulfill criteria for NHE1 being a \textit{bona fide} Akt substrate, including direct phosphorylation \textit{in vitro}, using mass spectrometry and Akt phospho-substrate antibodies to identify Ser\textsuperscript{648} as the Akt phosphorylation site and loss of increased exchanger phosphorylation and activity by insulin and platelet-derived growth factor in fibroblasts expressing a mutant NHE1-S648A. How Akt induces actin cytoskeleton remodeling to promote cell migration and tumor cell metastasis is unclear, but disassembly of actin stress fibers by platelet-derived growth factor and insulin and increased proliferation in growth medium are inhibited in fibroblasts expressing NHE1-S648A. We predict that other functions shared by Akt and NHE1, including cell growth and survival, might be regulated by increased H\textsuperscript{+} efflux.

The serine/threonine kinase Akt/protein kinase B functions as a convergence site of cues that signal through the lipid kinase phosphoinositide 3-kinase (PI 3-kinase),\textsuperscript{4} including activated integrin receptors, growth factor receptor tyrosine kinases, cytokine receptors, and G protein-coupled receptors. Akt confers signal relay from these upstream regulators to increase cell proliferation, cell survival, protein synthesis, and glycolytic flux (1). These actions contribute to the positive effects of Akt in cancer progression, and PI 3-kinase/Akt signaling is commonly aberrant in human tumors (2). Akt also regulates reorganization of the actin cytoskeleton, including disassembly of bundled actin stress fibers and formation of actin filament-rich membrane protrusions (3, 4), which likely enhance the positive effects of some Akt isoforms on cell migration (5) and tumor cell metastasis (6). However, compared with most cell processes regulated by Akt, our understanding of how Akt regulates cytoskeleton dynamics is least understood.

Akt generally phosphorylates substrates at an RXRXX(S/T) recognition motif (7), and in most instances Akt phosphorylation inhibits activity or function of the substrate. Akt promotes cell proliferation by phosphorylating and inhibiting the negative cell cycle regulators p21\textsuperscript{Cip1/WAF1} (8) and p27\textsuperscript{Kip1} (9–11) and Wee1 kinase (12). It promotes cell survival by phosphorylating and inhibiting the function of proapoptosis regulators, including Bad (13, 14) and forkhead transcription factors (15, 16), and it increases protein synthesis by phosphorylating and inhibiting the negative regulators of mTOR, tubersclerosis sclerosis protein 2 (17) and proline-rich Akt substrate of 40 kDa (18). Additionally, Akt increases metabolism in part by phosphorylating and inhibiting glycogen synthase 3 (19). However, there are a few exceptions with phosphorylation by Akt increasing substrate activity or function. Phosphorylation by Akt increases activity of the ubiquitin E3 ligase MDM2 (20), endothelial nitrogen-oxide synthase (21, 22), and the FYVE domain-containing protein 2 (17) and proline-rich Akt substrate of 40 kDa (23). Hence, substrates activated by Akt are exceptions rather than the general axiom.

We report that the ubiquitously expressed sodium-hydrogen exchanger isoform NHE1 is directly phosphorylated and activated by the Akt1 isoform. By catalyzing the electroneutral exchange of intracellular H\textsuperscript{+} for extracellular Na\textsuperscript{+}, NHE1 regulates intracellular pH and cell volume homeostasis. Increased NHE1 promotes cell proliferation (24, 25) and in migrating cells is necessary for polarity (26–28), directional movement (26, 27, 29), and \textit{de novo} actin filament assembly (27, 30). Like Akt, NHE1 also promotes cell survival, with NHE1 scaffolding (31) and activity (32) providing anti-apoptotic signals. H\textsuperscript{+} efflux by the transmembrane domain of NHE1 is regulated by post-
translational modifications of a C-terminal cytoplasmic regulatory domain, including phosphorylation of multiple serine residues. NHE1 is phosphorylated and activated by p90 ribosomal S6 kinase (p90Rsk) (33), Rho-activated kinase ROCK (34), p38 mitogen-activated kinase (p38 MAPK) (35), and the Ste20-like Nck-interacting kinase NIK (36). Although PI 3-kinase activity is necessary for increased NHE1 activity by insulin in erythrocytes (37) and by platelet-derived growth factor (PDGF) in Chinese hamster ovary cells (38), the serine/threonine kinase mediating activation by PI 3-kinase is undetermined. During completion of our study, Avkiran and co-workers (39) reported that Akt directly phosphorylates NHE1; however, their data in cardiomyocytes show that Akt phosphorylation decreases NHE1 activity. In contrast, we report that in fibroblasts Akt phosphorylation of NHE1 increases activity and is necessary for activation of NHE1 by insulin and PDGF. Moreover, increased H+ efflux by Akt phosphorylation of NHE1 is necessary for disassembly of actin stress fibers by insulin and PDGF and for cell proliferation in growth medium. The broad significance of these data includes identifying a substrate of Akt that is activated by phosphorylation, understanding growth factor regulation of NHE1 and pH, which is often aberrant in pathological conditions, and insight on the poorly understood mechanism whereby growth factors and Akt induce disassembly of bundled actin filaments.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—The mammalian expression constructs pCMV-NHE1-HA, containing the full-length rat NHE1 sequence 3′-end HA epitope tag (25), and pCAG-Myc-ROCKΔ3, containing a 3′-end truncated murine p160ROCK sequence with a 5′-end Myc tag (34) were described previously. The full-length cytoplasmic regulatory domain of human NHE1 (amino acids 503–815) and a C-terminal fragment (amino acids 638–815) from rabbit NHE1 were subcloned into pGEX vector (GE Healthcare, Piscataway, NJ) and affinity-purified using glutathione-agarose (BD Biosciences, San Jose, CA) as described (36). Serine to alanine substitutions were generated in pGEX-NHE1(638–815) (forward primers GCCGC GGCTGCGCCGCCTACAAACAGACAC for S648A and CCCG CATCGGCCCGACCCGCTGGGC for S703A) and pCMV-NHE1-HA (forward primers GCAACGCTGGCGGCCTAT AACAGACAC for S648A and GGCCGGCATAGGGCGCCGAC CCCACTGGCC for S703A) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol.

**Cells**—Wild-type and mutant NHE1-HA were stably expressed in NHE1-deficient PS120 cells, derived from CCL39 hamster lung fibroblasts as described (25). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated FBS (growth medium). HEK-293T cells were maintained in DMEM supplemented with 10% FBS, and NMuMG mouse mammary epithelial cells were maintained in DMEM supplemented with 10% FBS and 10 μg/ml insulin. Full-length wild-type and mutant NHE1-HA were stably expressed in PS120 cells by co-transfecting 10 μg of pCMV-NHE1-HA plasmids and 1 μg of pRSV-Neo using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. G418-resistant clones were subjected to an NH4Cl-induced acid load as described previously (25) as a second selection for NHE1-expressing cells based on H+ efflux. Expression of NHE1 was confirmed by immunoblotting cell lysates with a monoclonal NHE1 antibody (1:5000; Chemicon/Millipore, Billerica, MA), and by measuring pH recovery of an NH4Cl-induced acid load as described below. For transient expression of proteins, HEK-293T cells were transfected with 24 μg of DNA using Lipofectamine 2000 according to the manufacturer’s protocol and used 48 h after transfection.

**Kinase Assays**—In vitro kinase assays included 5 μg of myelin basic protein, GST alone, GST-NHE1(503–815), or wild-type and mutated GST-NHE1(638–815) as substrates incubated in buffer A (20 mM Tris-HCl, pH 7.5, 75 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol), 20 μM ATP, and 5 μCi of [γ-32P]ATP for reactions with Akt, protein kinase Ca, and ROCK and buffer B (25 mM Hepes, pH 7.5, 10 mM MgCl2, 3 mM MnCl2, 1 mM dithiothreitol, 1 mM Na3VO4, 10 μM ATP, and 5 μCi of [γ-32P]ATP) for reactions with NIK. Partially active or mutationally inactive (K179A) EE-tagged Akt1 was provided by David Stokoe (40), and His-tagged NIK kinase domain (amino acids 1–305) in baculovirus was expressed in Sf9 cells and precipitated using a nickel affinity column. Protein kinase Ca was purchased from Cell Signaling Technology (Beverly, MA). For ROCK, 293T cells transfected with pCAG-Myc-p160ROCKΔ3 were lysed in lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM EGTA, 5 mM NaF, 10 mM sodium pyrophosphate, 1 mM glycerol phosphate, 1 mM sodium vanadate, and Complete™ protease inhibitors mixture (Roche Applied Science)), precelled with protein G-Sepharose (GE Healthcare), and incubated for 1 h at 4 °C with Myc antibody (clone 9B11; Cell Signaling Technology). Protein G-Sepharose was added for 1 h at 4 °C, after which immune complexes were collected by centrifugation and washed three times in lysis buffer. Prior to the kinase assay, the beads were recollected and suspended in buffer A. Kinase reactions were maintained for 30 min at 30 °C and terminated by addition of sample loading buffer. The samples were separated by SDS-PAGE and stained with Coomassie dye, and phosphorylation was visualized by autoradiography. For immunodetection of Akt phosphorylated GST-NHE1, 100 ng of protein was incubated with Akt1 and unlabeled ATP and immunoblotted using a phospho-Akt-substrate antibody that recognizes the motif (R/K)Xp(S/T) (Cell Signaling Technology).

**Mass Spectrometry**—In-gel tryptic digestion of GST-NHE1(638–815) incubated with wild-type and mutationally inactive Akt was performed as described (41). Electrospray ionization liquid chromatography MS/MS analysis was as described (42) (supplemental Fig. S1) and employed an inclusion list that consisted of m/z values (2+ and 3+) for the theoretically predicted NHE1-derived Ser-, Thr-, and Tyr-containing peptides carrying up to one phosphate moiety. The in-house Mascot search engine (Matrix Science) was employed. Peak lists were generated by “Peaks-to-Mascot” script (Applied Biosystems Inc, Foster City, CA); the search was limited to doubly and triply charged precursors. Taxonomy mammalia (42,826 sequences) within SwissProt 50.0 (222,289 sequences) were interrogated using the following settings: enzyme Tryp...
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sin/P; fixed modifications: Cys-carbamidomethyl; variable modifications: N-acetyl (protein), deamidation (Asn and Gln), Met-sulfoxide, Pyro-glu (N-terminal Gln), phospho-(STY); mass: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ±250 ppm; fragment mass tolerance: ±0.2 Da; maximum missed cleavages: 3; instrument type: electrospray ionization-quadrupole-time-of-flight. Individual ions scores of ≥40 were indicative of identity or extensive homology (p < 0.05).

**NHE1 Phosphorylation in Cells**—The cells plated at 0.65 × 10⁶/100-mm dish were maintained in growth medium for 24 h, transferred to DMEM containing 0.2% FBS for 18 h, washed, maintained for 60 min in NaPO₄-free DMEM in the absence of FBS, and maintained then for 4 h in NaPO₄-free DMEM containing 500 µCi of [³²P]orthophosphate in 5 ml/dish. Insulin (100 nM) and PDGF (50 ng/ml) were added for the indicated times, and the cells were washed four times in cold phosphate-buffered saline, lysed in modified radioimmune precipitation assay buffer (50 mM Tris-HCl, 135 mM NaCl, 3 mM KCl, 1% Nonidet P-40, protease inhibitors, 1 mM EDTA, 5 mM NaF, 10 mM sodium pyrophosphate, 1 mM glycerol phosphate, 1 mM sodium vanadate, and 10 mM calyculin A, pH 7.4), and centrifuged at 800 × g for 5 min, and the supernatant was retained for immunoprecipitation of NHE1-HA by incubating for 18 h with Sepharose-conjugated anti-HA antibodies (Roche Applied Science). Immune complexes were recovered by centrifugation, separated by 7.5% SDS-PAGE, silver-stained, and dried, and [³²P]-labeled NHE1 was determined by autoradiography.

**NHE1 Activity**—NHE1 activity was determined as the rate of pH₄ recovery (dpH₄/dt) from an NH₄Cl-induced acid load as previously described (25) with modifications for using a plate reader to measure the fluorescence of cells loaded with the H⁺-sensitive dye 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Invitrogen). The cells plated at 2 × 10⁴/well in 24-well plates were maintained in growth medium for 24 h, transferred to DMEM containing 0.2% FBS for 18 h, and loaded with 1 µM BCECF in a nominally HCO₃⁻-free Hepes buffer (145 mM NaCl, 5 mM KCl, 10 mM glucose, 25 mM Hepes, 1 mM MgSO₄, 1 mM CaCl₂, pH 7.4) for 10 min at 37 °C in the absence of CO₂. The cells were washed with Hepes buffer and incubated for 10 min in 30 mM NH₄Cl. Growth factors or NaCl (for osmotic activation) were added for 1 min, and NH₄Cl was rapidly aspirated and replaced with Hepes buffer containing the indicated concentrations of growth factors or NaCl. For studies with pharmacological inhibitors, the reagents were added to the BCECF, NH₄Cl, and Hepes recovery buffers for a 20-min preincubation before measuring NHE1 activity. Ratios of BCECF fluorescence at Ex₄90/Em₅30 and Ex₄40/Em₅30 were acquired every 15 s for 5 min using a SpectraMax M5 plate reader (Molecular Dynamics, Sunnyvale, CA). The fluorescence ratios were converted to pH₄ by calibrating each well with 10 µM nigericin (Invitrogen) in 105 mM KCl as described (25). NHE1 activity was expressed as dpH₄/dt by evaluating the derivative of the slope of the time-dependent pH₄ recovery at intervals of 0.05 pH units using a program developed by Duncan Wong (Yale University). Statistical analyses were performed using GraphPad software with n values representing the number of separate cell preparations.

**Immunoblotting**—The cells grown to subconfluence in 6-well plates were maintained overnight in DMEM supplemented with 0.2% FBS, pretreated for 20 min with 10 µM LY294002 (Sigma-Aldrich), 10 µM U0126 (Calbiochem, San Diego, CA), 10 µM Akt X (Calbiochem), 100 nM rapamycin (Calbiochem), or Me₂SO as vehicle control and treated with insulin (100 nM; Sigma-Aldrich) or PDGF (50 ng/ml; Roche Applied Science). The cells were lysed in ice-cold lysis buffer and centrifuged at 800 × g for 5 min, and 10 µg of postnuclear supernatants were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes for immunoblotting. The blots were probed with antibodies to p44/p42 MAPK (Erk1/2, 1:1000) phosphorylated Erk1/2 (Thr(P)202/Tyr204, 1:1000), Akt (1:1000), phosphorylated Akt (Ser(P)473, 1:1000) (all from Cell Signaling Technology), phosphorylated ribosomal protein 6 (Santa Cruz Biotechnologies), α-tubulin (1:10,000; Sigma), or β-actin (clone C4, 1:10,000; Chemicon/Millipore). Bound antibody was detected by enhanced chemiluminescence.

**F-actin Labeling**—To determine F-actin organization, quiescent cells plated on glass coverslips were treated for 10 min with insulin (100 nM) or PDGF (50 ng/ml), washed in phosphate-buffered saline, and fixed in 3% paraformaldehyde. After washing, the cells were permeabilized by incubating for 10 min in 0.1% Triton X-100 in phosphate-buffered saline, washed, and incubated for 30 min with rhodamine-phalloidin (1:5,000; Invitrogen). The images were acquired using a spinning disk confocal (Yokogawa; CSU-10) on a microscope (Nikon; TE-2000) with a 100× objective (Plan TIRF 1.49 NA) equipped with a cooled CCD camera (Photometrics; CoolSnap HQ2) and analyzed using NIS-Elements software (Nikon).

**RESULTS**

**Akt Directly Phosphorylates NHE1**—We asked whether NHE1 might be an Akt substrate because it is a regulator of Akt kinase activity in response to apoptotic stress (31), and it contains two RXRXX(S/T) motifs in the distal cytoplasmic regulatory domain (Fig. 1A). Using an in vitro kinase assay, we confirmed that purified Akt1, and NIK as previously described (36), phosphorylated a GST fusion of the full-length cytoplasmic regulatory domain of NHE1 (amino acids 503–815) but not GST alone (Fig. 1B). NHE1 was not phosphorylated by a mutant kinase-inactive Akt (K179A) or by heat-inactivated NIK. Specificity of kinase phosphorylation of NHE1 was confirmed by purified protein kinase C phosphorylating myelin basic protein but not GST-NHE1 (Fig. 1C).

A GST fusion of the NHE1 distal cytoplasmic domain (amino acids 638–815) that contains all predicted NHE1 phosphorylation sites was used to identify the Akt1 phosphorylation site(s) by liquid chromatography-MS/MS. Analysis of tryptic peptides (Fig. 1D) revealed a double charged ion m/z 444.7 in the sample treated with wild-type Akt1 (top panel) but not with mutant kinase inactive Akt1 (bottom panel). The MS/MS spectrum of this ion was typical for the collisionally induced fragmentation of a phosphorylated peptide; the major product ions were derived from the neutral loss of H₂PO₄⁻ (~98 Da) and HPO₃⁻ (~80 Da) from the precursor ion, whereas sequence-specific product ions were seen at low intensities (supplemental Fig. S1) (43). Direct assignment of the phosphorylated residue could
However, extensive neutral losses observed in MS/MS point to Ser\textsuperscript{648} (rabbit NHE1) rather than an adjacent Tyr\textsuperscript{649} as the site of phosphorylation. The sequence surrounding Ser\textsuperscript{648} confers to the protein kinase B consensus motif R\textsuperscript{X}R\textsuperscript{XX}(S/T). A second protein kinase B consensus motif is present in the regulatory domain at Ser\textsuperscript{703} (Fig. 1A) that is phosphorylated by p90Rsk (33). The serine in this motif, however, is followed by an acidic residue (aspartic acid), which makes it unfavorable for phosphorylation by Akt (7). Ser\textsuperscript{648} was recognized by Scansite as an Akt phosphorylation site in a high stringency scan (0.142%), but Ser\textsuperscript{703} was recognized only in a medium stringency scan (0.464%).

We confirmed Ser\textsuperscript{648} but not Ser\textsuperscript{703} as the site of Akt phosphorylation by using GST-NHE1(638–815) wild-type and S648A and S703A mutants in an \textit{in vitro} kinase assay. Phosphorylation by Akt\textsubscript{1} was similar with wild-type NHE1 and NHE1-S703A but was not seen with NHE1-S648A (Fig. 2A). In contrast, wild-type NHE1, NHE1-S648A, and NHE1-S703A were phosphorylated by a Myc-tagged constitutively active ROCK (ROCK\textsubscript{Δ3}) transiently expressed in 293T cells and immunoprecipitated (Fig. 2A) and by purified recombinant NIK (Fig. 2B). To further confirm that Ser\textsuperscript{648} is the amino acid phosphorylated by Akt\textsubscript{1}, we immunoblotted Akt- phospho- in an in vitro kinase assay with 20 μM [γ\textsuperscript{32}P]ATP for Akt or 10 μM [γ\textsuperscript{32}P]ATP for NIK and 2–5 μG of GST or GST-NHE1 (503–815) as substrates and incubated for 30 min at 30 °C. Autoradiograph (autorad; left) and Coomassie stain (coomassie; right) of kinase assay. C, kinase assay with 100 ng of purified recombinant protein kinase C\textsubscript{α}, 20 μM [γ\textsuperscript{32}P]ATP, and 2 μG of GST, GST-NHE1 (638–815), or myelin basic protein as in A. Top panel, autoradiography; bottom panel, Coomassie stain of kinase assay. D, MS identification of the Akt\textsubscript{1} phosphorylation site in NHE1. Phosphorylated peptide \textsuperscript{646}LRS(phospho)YN\textsuperscript{651}R (doubly charged ion at \textit{m/z} 444.7 marked with arrow) is seen in the mass spectrum of species derived from GST-NHE1(638–815) that was treated with wild-type Akt\textsubscript{1} (top panel) but not in the corresponding mass spectrum of sample treated with mutationally inactive Akt\textsubscript{1} (bottom panel). Mass spectra of both samples represent the apex of elution of an ion 446.2 that is co-eluting with the phosphorylated peptide. The identity of ion \textit{m/z} 444.7 was authenticated by MS/MS analysis (supplemental Fig. S1).
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NHE1-S703A cells. With PDGF (50 ng/ml; 10 min), increased phosphorylation was greater in 85-kDa NHE1 than in 98-kDa NHE1 in WT cells but was seen only in 98-kDa NHE1 in NHE1-S703A cells, although the reason for these differences is unknown. Together with our in vitro data, our findings indicate the physiological significance of Ser648 but not Ser703 for phosphorylation of NHE1 by Akt, insulin, and PDGF.

Phosphorylation of Ser648 but Not Ser703 Is Necessary for Increased NHE1 Activity with Insulin and PDGF—Because increased phosphorylation of NHE1 is generally associated with stimulating NHE1 activity, and insulin and PDGF activate Akt, we asked whether phosphorylation of Ser648 is necessary for increased NHE1 activity by insulin and PDGF. In WT cells, pH4-dependent NHE1 activity, determined as the rate of pH4 recovery (dPH4/dt) from an NH4Cl-induced acid load, was greater with insulin (100 nM) and PDGF (50 ng/ml) than in quiescent controls (supplemental Fig. S2 and Fig. 4, A and C). Maximal activity at pH4 6.6 significantly increased from 65.9 ± 6.1 × 10−4 PH/s in controls to 98.6 ± 7.4 × 10−4 PH/s with insulin (p < 0.01, n = 5) and to 98.7 ± 6.3 × 10−4 PH/s with PDGF (p < 0.01, n = 5). Additionally, the set point for quiescent NHE1 activity increased from approximately pH4 7.2 to pH4 7.4 with insulin (Fig. 4A) and to pH 7.5 with PDGF (Fig. 4B). In NHE1-S648A and NHE1-S703A cells, control NHE1 activity was not different from that of WT cells (Fig. 4, A and B). Insulin and PDGF increased NHE1 activity in NHE1-S703A cells similar to that in WT cells; however, activity did not increase in NHE1-S648A cells (Fig. 4, A–D). Similar data were obtained in two different clones of NHE1-S648A and NHE1-S703A cells (data not shown). In contrast, thrombin (30 nM), which like lysophosphatidic acid acts through ROCK phosphorylation of NHE1 (34), increased activity in WT, NHE1-S648A, and NHE1-S703A cells, with no significant differences in maximal activity between the three cell types (Fig. 4, E and F; p > 0.5, n = 6). Increased osmolarity increases NHE1 activity independent of increased NHE1 phosphorylation (45), and acute addition of 100 mM NaCl in the pH4 recovery buffer stimulated NHE1 activity to a similar extent in all three cell types (supplemental Fig. S3, A and B).

Phosphorylation of Ser703 is necessary for increased NHE1 activity with serum (33), which we confirmed by showing that acute addition of 5% FBS to the pH4 recovery buffer stimulated NHE1 activity in WT cells but not in NHE1-S703A cells (Fig. 5). However, FBS also did not increase the activity of NHE1-S648A cells (Fig. 5). Taken together, these data indicate that phosphorylation of Ser648 is necessary for increased NHE1 activity with insulin, PDGF, and FBS but not with thrombin or hyperosmolarity and that phosphorylation of Ser703 is necessary for increased NHE1 activity with FBS but not with insulin, PDGF, thrombin, or hyperosmolarity. Although the NHE1 site phosphorylated by ROCK remains undetermined, our in vitro kinase data (Fig. 2A) and our functional data (Fig. 4, E and F), suggest that the residue is not Ser648 or Ser703.

Inhibition of PI 3-Kinase and Akt but Not MEK Blocks Increased NHE1 Activity with Insulin and PDGF—We also found that increased NHE1 activity by insulin and PDGF is dependent on a PI 3-ki-
nase/Akt signaling pathway. Pretreating WT cells for 20 min with LY294002 (10 μM), which inhibits PI 3-kinase activity, or with Akt X (10 μM), which inhibits Akt activity (46), had no effect on control NHE1 activity but completely blocked increased activity with insulin (Fig. 6, A and C) and with PDGF (Fig. 6, B and D). In contrast, pretreatment with U0126 (10 μM), which inhibits MEK activity, did not significantly attenuate control or growth factor-stimulated NHE1 activity (Fig. 6, E and F). Maximal activity at pH\textsubscript{i} 6.6 in the absence (Me2SO vehicle control) and presence of U1026, respectively, was 72.7 × 10^{-4} pH/s for controls (p = 0.5, n = 4), 98.7 ± 7.41 and 87.2 ± 3.2 × 10^{-4} pH/s with insulin (p > 0.5, n = 4), and 98.6 ± 6.2 and 89.8 ± 3.4 × 10^{-4} pH/s with PDGF (p > 0.5, n = 4). Additionally, insulin-stimulated NHE1 activity in NMuMG mammary epithelial cells was blocked by AktX but not by U0126 (supplemental Fig. S4, A and B). In contrast to growth factor activation of NHE1, osmotic activation was not attenuated by LY294002, Akt X, or U0126 (supplemental Fig. S4, C–F). Previous findings also showed that increased NHE1 activity with hyperosmolarity is not blocked by inhibiting the activity of ERK (47).

Immunoblotting cell lysates confirmed the efficacy of inhibitors. LY294002 completely inhibited insulin (Fig. 6G) and PDGF (Fig. 6H) phosphorylation of Akt (pAkt) but not phosphorylation of ERK (pERK1/2). In contrast, U0126 selectively blocked increased pERK1/2 by both growth factors without affecting increased abundance of pAkt (Fig. 6, G and H). The effects of Akt X were less specific. With insulin, 5 μM Akt X completely blocked pAkt and reproducibly increased pERK1/2 (p = 0.5, n = 3), although the reason for this increase is unknown. With PDGF, 10 μM Akt X was necessary to inhibit pAkt, but this concentration also attenuated the abundance of pERK1/2 (Fig. 6F). Hence, although insulin and PDGF activate both PI 3-kinase/Akt and MEK/ERK signaling modules, our data suggest that their stimulation of NHE1 activity is mediated by PI 3-kinase and Akt.

To further support our findings that Akt directly phosphorylates NHE1 in cells, we tested the prediction that activity of a downstream Akt effector should not be necessary for stimulation of NHE1 by insulin. We confirmed that pretreating cells with rapamycin (100 nM, 20 min), which inhibits mTOR, a downstream effector of Akt, had no effect on quiescent or insulin-stimulated NHE1 activity (Fig. 7A). Immunoblotting for phosphorylated S6 ribosomal protein, which increases with stimulated mTOR activity, confirmed the increased abundance with insulin that was completely inhibited with rapamycin (Fig. 7B).

The Akt Phosphorylation Site in NHE1 Is Necessary for Regulated Disassembly of Actin Stress Fibers and for Cell Proliferation—To determine the functional significance of the Akt phosphorylation site in NHE1 beyond increasing exchanger activity, we first tested actin cytoskeleton organization, which is regulated by PDGF (48, 49), insulin (50, 51), Akt (3), and NHE1 (25). These studies also included cells expressing a mutant inactive NHE1-E266I that lacks H\textsuperscript{+} efflux in the absence and presence of growth factors (25, 28). The abundance and organization of actin filaments, determined by rho-
damine-phalloidin labeling, were similar in quiescent cells expressing wild-type or mutant NHE1 (Fig. 8A). Actin stress fibers in the cell body were predominantly in parallel arrays near the basal membrane and cortical actin filaments extended into angular protrusions. Because NHE1-E266I is inactive, these data indicate that H\(^+\) extrusion by NHE1 is not necessary for the organization or assembly of actin stress fibers in quiescent cells, which is consistent with previous findings (25). WT cells treated with PDGF or insulin for 10 min had a marked reorganization of actin filaments, including disappearance of actin stress fibers in the cell center and appearance of F-actin clusters at rounded membrane protrusions (Fig. 8A and B). A similar reorganization was seen in NHE1-S703A cells treated with PDGF or insulin (Fig. 8A). In contrast, there was no detectable change in actin filament organization in NHE1-E266I cells or in NHE1-S648A cells treated with PDGF or insulin (Fig. 8A). The cells retained abundant parallel arrays of actin stress fibers in the cell body and cortical actin filaments aligned with the plasma membrane. NHE1-E266I and NHE1-S648A cells also retained angular membrane protrusions similar to quiescent cells and distinct from rounded protrusions in WT and NHE1-S703A cells treated with growth factors (Fig. 8B). Additionally, NHE1-S648A cells appeared larger or more spread than WT cells. These data suggest that increased NHE1 activity with PDGF and insulin, which occurs in WT and NHE1-S703A cells but not in NHE1-E266I or NHE1-S648A cells, is necessary for regulated disassembly of actin stress fibers and assembly of cortical F-actin structures.

We also tested for functional significance of the S703A mutation to support our finding that phosphorylation of Ser\(^{703}\) is necessary for increased NHE1 activity with FBS (Fig. 5B). NHE1 activity is recognized as a permissive signal for cell proliferation, and we previously showed that the proliferation rate of NHE1-E266I cells is ~4-fold less than WT cells (25). Consistent with increased H\(^+\) efflux by NHE1 promoting cell proliferation, in growth medium containing 5% FBS, proliferation of NHE1-S703A and NHE1-S648A cells was markedly less than WT cells or CCL39 cells (Fig. 8C), which are the parental cells used for generating the NHE1-deficient PS120 cells we used for expression of wild-type and mutant NHE1. Because quiescent NHE1 activity is not attenuated in NHE1-S703A and NHE1-S648A
cells, these data suggest a specific role for stimulated increases in NHE1 activity for cell proliferation.

**DISCUSSION**

Our current data on NHE1 fulfill many criteria outlined by Manning and Cantley (1) for defining a *bona fide* Akt substrate. We show that Akt directly phosphorylates the C-terminal regulatory domain of NHE1, which contains two RXRXX(S/T) motifs. Mass spectrometry identified the predominant Akt phosphorylation site as Ser$^{648}$ but not Ser$^{703}$, and the phosphorylation site mutant S703A but not S648A is phosphorylated by Akt in vitro and by PDGF and insulin in cells. Immunoblotting with Akt phospho-substrate antibodies confirms a requirement for Ser$^{648}$ but not Ser$^{703}$. Additional criteria fulfilled include showing a specific readout of the phosphorylation event in question, as indicated by loss of growth factor activation of NHE1-S648A but not NHE1-S703A, and selective pharmacological inhibition of growth factor-stimulated NHE1 activity by the PI 3-kinase inhibitor LY294002 and the Akt inhibitor Akt X but not by the MEK inhibitor U0126 or by rapamycin, an inhibitor of the Akt effector mTOR. Moreover, we also reveal functional significance of Akt phosphorylation of NHE1, which is not included as a criterion for confirming substrate identity. We show that disassembly of actin stress fibers by PDGF and insulin is inhibited in cells expressing NHE1-S648A that cannot be phosphorylated by Akt.

As an Akt substrate, NHE1 is notable in two respects. First, phosphorylation by Akt stimulates NHE1 activity. With few exceptions, phosphorylation by Akt generally inhibits substrate activity or function (1). During the completion of our study Avkiran and co-workers (39) also found that Akt phosphorylates NHE1 at Ser$^{648}$, but in myocardial cells used in their study, Akt inhibits NHE1 activity. However, in cardiomyocytes insulin

**FIGURE 7.** Rapamycin does not inhibit NHE1 activity. A, pH$_i$-dependent NHE1 activity in WT cells in the absence (control) and presence of insulin (100 nM) with and without rapamycin (100 nM). The data represent the means of three separate cell preparations. B, immunoblot with antibodies to phosphorylated rS6 (prS6) and α-tubulin of lysates from cells untreated or treated with rapamycin in the absence or presence of insulin. DMSO, dimethyl sulfoxide.

**FIGURE 8.** The Akt phosphorylation site in NHE1 is necessary for regulated disassembly of actin stress fibers and for cell proliferation. A, representative confocal images of the indicated cells types at quiescence (control) or treated for 10 min with PDGF (50 ng/ml) or insulin (100 nM) and labeled with rhodamine-phalloidin. The arrows indicate F-actin clustered at rounded membrane protrusions. The scale bar equals 5 μM. B, higher magnification of confocal images from A for WT and NHE1-S648A cells in the absence and presence of PDGF. The scale bar equals 100 μM. C, proliferation of the indicated cell types maintained in growth medium containing 5% FBS. The data are expressed as the number of cells for each cell type as a percentage of cell number at 24 h and are the means ± S.E. of four cell preparations.
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increases NHE1 activity (44); hence it remains to be determined what upstream cues control Akt inhibition of NHE1 in these cells. We found that insulin increases NHE1 activity in mammary epithelial cells, and this is blocked by pharmacological inhibition of Akt but not MEK. In other cell types insulin and PDGF increase NHE1 activity (28, 37, 38, 52, 53), although a role for Akt has not been reported.

Also notable for NHE1 as an Akt substrate is our finding that increased NHE1 activity is necessary for actin stress fiber disassembly by PDGF and insulin. Disassembly of actin stress fibers by PDGF (48, 49) and insulin (50, 51) has been recognized for many years, as has a role for increased activity of PI 3-kinase (51, 54) and Akt (3). However, of the many cell processes regulated by Akt, mechanisms mediating effects on actin filament organization are the most ill-defined. The Akt substrate Girdin/Akt-phosphorylation enhancer, an actin cross-linking protein, promotes assembly but not disassembly of actin stress fibers in response to epidermal growth factor stimulation of Vero cells (55). Increased activity of ribosomal protein S6 kinase p70S6K, an mTOR effector, promotes stress fiber disassembly (3, 56) but also stress fiber assembly in response to thornado (57). Perhaps the most compelling mediator is the Akt substrate and 14-3-3-binding protein Kank, which inhibits RhoA activity and induces actin stress fiber disassembly in fibroblasts (4). Disassembly of actin stress fibers by insulin is reported to be mediated by the mitogen activated protein kinase p38 (58) or by phosphatidylinositol 5-phosphate (59), whereas PDGF actions are reported to require p41/42 activity (60) or an E3 ubiquitin ligase specific for myosin regulatory light chain (61). Hence, how Akt signaling induces disassembly of actin stress fibers and whether this involves multiple nonredundant mechanisms are unresolved questions.

A necessary role of H⁺ efflux by NHE1 for stress fiber disassembly is functionally consistent with abundant stress fibers not being conductive to cell migration, and NHE1 activity is necessary for directed migration of fibroblasts. In migrating fibroblasts, focal adhesion remodeling (29, 62), cell polarity (26, 28), and de novo actin polymerization (30) are dependent on increased H⁺ efflux by NHE1. However, NHE1 binding to the ERMs proteins ezrin, radixin, and moesin but not NHE1 activity is necessary for RhoA-mediated assembly of actin stress fibers (25). Together these data suggest distinct actions of scaffolding and H⁺ efflux by NHE1 in the assembly and disassembly, respectively, of stress fibers. A paradox, however, is that disassembly of stress fibers by growth factors is associated with disassembly of focal adhesions, but the latter is inhibited by loss of NHE1 activity (62). Another paradox is that increased activity of the RhoA-kinase ROCK induces actin stress fiber assembly, which requires ROCK phosphorylation of NHE1 (34). We speculate that H⁺ efflux by NHE1 might be necessary for an active process disassembling stress fibers, such as regulating the Rho family protein Rnd3/RhoE that promotes stress fiber disassembly (63, 64). RhoE is a reasonable but unreported link between PDGF/insulin, Akt, and actin stress fiber disassembly.

In addition to cytoskeleton reorganization, other functions shared by Akt and NHE1 might be regulated by increased H⁺ efflux. Akt and NHE1 activity promote cell proliferation in part by inhibiting Wee1 kinase (12, 65), a negative regulator of Cdk1 and G₂/M progression. We confirmed that the Ser⁶⁴⁸ site phosphorylated by Akt is necessary for proliferation in growth medium, and an important future question to address is the importance of phosphorylated Ser⁶⁴⁸ in G₂/M transition and in Akt-regulated cell proliferation. Also of importance is our finding that although quiescent activity of NHE1-S648A and NHE1-S703A cells is similar to that of WT cells, their proliferation is markedly decreased. We previously showed an NHE1-dependent rapid and transient increase in pH₇ at the end of the S phase that times entry into G₂/M (66), and our current data suggest that phosphorylation of Ser⁶⁴⁸ and Ser⁷⁰³ may be necessary for this to occur.

As previously recognized (31), NHE1 functions in Akt-dependent cell survival, determined by NHE1 scaffolding (31) and activity (32), and decreased pH₇ is a recognized pro-apoptotic signal (67). Increased H⁺ efflux by NHE1 also could be a determinant in Akt promoting cancer progression because increased pH₇ is a hallmark of most tumor cells and correlates with metastatic potential (68, 69). Our findings highlight several questions for future investigation, including the paradox of Akt phosphorylation of NHE1 being inhibitory in cardiomyocytes but stimulatory in fibroblasts and the functional significance of H⁺ efflux by NHE1 in myriad normal and pathological processes regulated by Akt.

Acknowledgments—We thank David Stokoe for valuable suggestions and reagents, Torsten Wittmann for help with confocal microscopy, and Scott Dixon for assistance with MS analysis.

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