Apoptosis results in cell shrinkage and intracellular acidification, processes opposed by the ubiquitously expressed NHE1 Na⁺/H⁺ exchanger. In addition to mediating Na⁺/H⁺ transport, NHE1 interacts with ezrin/radixin/moesin (ERM), which tethers NHE1 to cortical actin cytoskeleton to regulate cell shape, adhesion, motility, and resistance to apoptosis. We hypothesize that apoptotic stress activates NHE1-dependent Na⁺/H⁺ exchange, and NHE1-ERM interaction is required for cell survival signaling. Apoptotic stimuli induced NHE1-regulated Na⁺/H⁺ transport, as demonstrated by ethyl-N-isopropyl-amiloride-inhibitable, intracellular alkalinization. Ectopic NHE1, but not NHE3, expression rescued NHE1-null cells from apoptosis induced by staurosporine or N-ethylmaleimide-stimulated KCl efflux. When cells were subjected to apoptotic stress, NHE1 and phosphorylated ERM physically associated within the cytoskeleton-enriched fraction, resulting in activation of the pro-survival kinase, Akt. NHE1-associated Akt activity and cell survival were inhibited in cells expressing ERM binding-deficient NHE1, dominant negative ezrin constructs, or ezrin mutants with defective binding to phosphoinositide 3-kinase, an upstream regulator of Akt. We conclude that NHE1 promotes cell survival by dual mechanisms: by defending cell volume and pH, through Na⁺/H⁺ exchange and by functioning as a scaffold for recruitment of a signalplex that includes ERM, phosphoinositide 3-kinase, and Akt.

Apoptosis is necessary for organogenesis, resolution of tissue injury, and pathobiologic cell deletion programs. Typical morphologic features of apoptotic cells include membrane blebbing, which is invariably followed by cytoplasmic shrinkage and chromatin condensation. Pathways regulating apoptotic volume decrease (AVD) have been extensively studied and include activation of ion channels that mediate Na⁺, K⁺, and Cl⁻ efflux (1). However, less is known about counter-regulatory pathways, which promote cell survival by maintaining intracellular volume.

In contrast to lymphocytes and mesenchymal cells, epithelial cells are relatively resistant to shrinkage from hypertonic stimuli or AVD (2, 3), because of robust intracellular volume expansion through activation of regulatory volume increase (RVI) pathways (4–7). Activation of the ubiquitously expressed NHE1 isoform of the Na⁺/H⁺ exchanger is an important RVI component (5–7), resulting in Na⁺ influx and cytoplasmic volume expansion. Concomitant NHE1-dependent H⁺ extrusion also leads to intracellular alkalinization, which may defend against apoptosis by inhibiting caspase catalysis (8, 9); by preventing conformational changes in Bax, a pro-apoptotic Bcl-2 family member (10); or by extruding cytosolic H⁺ resulting from apoptosis-induced mitochondrial H⁺ release (8).

We previously demonstrated that NHE1 is cleaved by caspase-3 in renal tubular epithelial cell apoptosis, and apoptosis in an NHE-deficient cell line was diminished by NHE1 reconstitution (11), indicating that NHE1 activity is critical for cell survival. Furthermore, mutant NHE1 expression studies demonstrated that domains regulating Na⁺/H⁺ exchange, as well as interaction with ezrin/radixin/moesin (ERM) proteins, were required for cell survival (11). ERM proteins bind directly to the membrane-proximal NHE1 cytoplasmic domain (12), which tethers NHE1 to the cortical actin cytoskeleton and regulates Na⁺/H⁺ transport-independent functions, such as maintenance of cell shape, focal adhesion formation, and specific aspects of cell migration (12, 13), implying that NHE1 directs separable Na⁺/H⁺ exchange- and ERM-dependent cell functions.

Linkage of NHE1 and ERM to downstream cell survival signaling pathways has not been described. ERM proteins were originally identified as molecular linkers between cytoskeleton and plasma membrane proteins and have therefore been viewed primarily as structural proteins. However, ezrin has subsequently been shown to directly interact with signaling enzymes, such as phosphoinositide 3-kinase (PI3K) (14–16), suggesting that ERM proteins may also regulate cell phenotype by functioning as molecular scaffolds for assembly and integration of cytoskeleton-based signalplexes. Because PI3K phosphorylates diverse protein substrates, including precursors in a cascade that results in activation of the survival kinase Akt, we have hypothesized that cell survival is promoted by a signaling complex that includes NHE1, ERM, PI3K, and Akt. We show that apoptotic or hypertonic stress causes NHE1-mediated Na⁺/H⁺ exchange, as well as activation of a new pathway that involves NHE1 interaction with ERM proteins within a cytoskeletal compartment, which up-regulates Akt activity and cell survival.
NHE1 Recruits ERM for Cell Survival

EXPERIMENTAL PROCEDURES

Materials—(6-Diamidino-2-phenylindole (DAPI), ethyl-N-isopropyl-[3H]pyrrolidinomethyl (EIPA), staurosporine (STS), N-ethylmaleimide (NEM), NHE1 antibodies, Wortmannin, and α-tubulin antibodies (Sigma), Akt inhibitor and LY294002 (Calbiochem, San Diego, CA); 2',7'-bis (2-carboxyethyl)-5 (6-carboxyfluorescein) (BCECF)-acetyloxyethyl ester and nigericin (Molecular Probes, Eugene, OR); annexin V-Alexa568, anti-hemaggutinin (HA) IgG, and Fugene 6 transfection reagent (Roche Applied Science); anti-phospho-Akt and -ERK1/2 (Cell Signaling Technology); anti-EZrin, AT-11, and Myc-tagged antibodies (Beverly, MA); monoclonal antibodies (Lab Vision Corporation, Fremont, CA); and anti-Akt-1 monoclonal antibodies and horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Rabbit polyclonal anti-NHE1 IgG was generated against an NHE1 cytoplasmic domain peptide (17) and affinity-purified as previously described (11). Carboxyl-terminal, HA epitope-tagged rat NHE1 cDNA was a gift from Dr. J. Orlovski (McGill University). Anti-NHE3 IgG was a gift from Dr. O. Moe (University of Texas Southwestern, Dallas, TX). K/RA and E2661 NHE1 mutant cDNAs were gifts from Dr. D. Barber (University of California at San Francisco).

Cell Lines—The HRPT human renal proximal tubule epithelial cell line (a gift from Dr. L. Racusen, Johns Hopkins University) and LLC-PK1 cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium with Ham's F-12 medium (Invitrogen) plus 10% fetal bovine serum (HyClone, Logan, UT) and 0.7 g/liter G418 (Invitrogen). In some experiments, the blots were stripped with 100 mM SDS-PAGE buffer (125 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 1% β-mercaptoethanol, and 0.003% bromphenol blue) and assayed for protein content (Bio-Rad). The proteins were denatured by boiling for 5 min, and the samples were resolved by 8%–20% gradient SDS-PAGE (Novex, San Diego, CA). The proteins were transferred to polyvinylidene difluoride membranes, blocked with 5% nonfat milk, and incubated with anti-HA (for NHE1 expression), anti-phospho-ERM, or anti-phospho-Akt antibody (1:1000, 4°C, overnight), followed by horseradish peroxidase-conjugated anti-rabbit (1:2000 for 1 h at room temperature) or anti-mouse (1:14,000, 1 h, room temperature) antibodies. In some experiments, the blots were stripped with 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8, and reprobed for protein loading with anti-actin (1:500 at 4°C overnight) or anti-Akt1 (1:100, 4°C, overnight) antibodies. Protein bands were detected by enhanced chemiluminescence (Amersham BioSciences). In some experiments, individual band intensity was quantified using Adobe Photoshop software (San Jose, CA).

Immunoprecipitation from Cytoskeleton Fractions—The cells were lysed in buffer containing 1% Nonidet P-40, 0.5% deoxycholic acid, 0.05% SDS, 50 mM HEPES, 135 mM NaCl, 3 mM KCl, 1 mM EDTA, and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) (4°C, 1 h). The lysates were centrifuged at 12,000 rpm for 30 min at 4°C. Cytoskeleton-rich pellets were resuspended in 100 μl of 1% SDS and then diluted 1:10 in lysis buffer. The protein content was quantitated, and lysates containing 700 μg of protein were immunoprecipitated with anti-NHE1 antibodies (0.5 μg/ml, 4°C, overnight) according to established methods (24). The samples were incubated with γ-bind protein G-Sepharose beads (4°C for 1 h) and then washed with lysis buffer. Immunoprecipitates were dissolved in 25 μl of 2× SDS sample buffer, resolved by 4–20% SDS-PAGE, and then evaluated as described under "Immunoblot Analysis.

RNA Interference—Small interfering RNA (siRNA) inhibition of Akt expression was achieved with the SignalSilence Akt siRNA kit according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA). Briefly, LLC-PK1 cells at 50% confluence were incubated with Akt siRNA, negative control (24), or fluorescein-labeled, nonspecific siRNA (to assess transfection efficiency) (50 nM each). At 48 h post-transfection, transfection efficiency was verified to be >90%. The cells were then induced to undergo apoptosis, lysed, and probed for Akt expression by immunoblots. The blots were stripped and reprobed for α-tubulin as a control for protein loading and nonspecific RNA interference effects. In parallel experiments, siRNA-transfected cells were assayed for apoptosis by annexin V labeling and DAPI staining of chromatin, as described above.

Statistics—The data are representative of three experiments/condition. The graphical results are expressed as the means ± S.E. Comparisons between groups were made by one-way analysis of variance with the Student-Newman-Keuls or Kruskal-Wallis test for multiple comparisons of parametric and nonparametric data, respectively. Comparisons between two groups were made by paired t test methods. Statistical significance is defined as p < 0.05.

RESULTS

NHE1 Defends against Apoptotic Stress—To assess whether NHE1 is activated in cells subjected to apoptotic stress, HRPT cells were stimulated with staurosporine in the presence and absence of NHE1 selective inhibitor EIPA. Intracellular pH was determined by BCECF-acetyloxyethyl ester fluorescence. HRPT cells demonstrated initial intracellular alkalization in response to staurosporine, which was blunted by EIPA (Table I), suggesting that NHE1-dependent RVI is a defense against apoptosis. Staurosporine exposure beyond 2 h was associated with cytosol acidification (Table I), in agreement with the kinetic of NHE1 degradation by caspase-3 (11). Similar results were observed following UV light exposure (30 J/m²), a dissimilar stimulus of mitochondrial apoptosis (not shown). These data are consistent with a recent report by Bortner and Ci-Rulowski (25), which shows that apoptotic stress induces a transient increase and then a profound decrease in Na⁺.

To examine the specificity of NHE1 in cell survival, NHE-deficient PS120 cells were transiently transfected to express NHE1 or NHE3 and then assessed for sensitivity to staurosporine-induced apoptosis. Fig. 1 shows that NHE1 expression resulted in ~50% decrease in apoptosis, consistent with our

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prior report (11). Unlike NHE1, ectopic NHE3 expression did not rescue PS120 cells from apoptosis. These data are in agreement with observations that cell shrinkage stimulates NHE1 activity, whereas NHE3 activity is decreased (26–28).

**NHE1 Rescues Cells from KCl Efflux-stimulated Apoptosis**—Many apoptosis models predict irreversible commitment to cell death following AVD, which is characterized by intracellular K⁺ and Cl⁻ loss, even in the presence of counter-regulatory NHE1 activity (7). To address this issue, NHE-null PS120 cells, transfected with NHE1 cDNA, were incubated with NEM, a thiol compound that activates K⁺/Cl⁻ co-transport (29), to induce K⁺ and Cl⁻ efflux. Fig. 2A demonstrates that NEM caused apoptosis, with a threshold effect at ~10 μM, a concentration that diminished K⁺ (not shown). Importantly, Fig. 2B shows that NHE1 expression significantly blunted NEM-mediated apoptosis, consistent with stoatrine data from Fig. 1. These studies demonstrate that NHE1 is important for cell survival, because activation was sufficient to block AVD, even under circumstances associated with robust K⁺ and Cl⁻ efflux.

**NHE1 Stimulation Leads to ERM Activation**—To explore the role of ERM proteins in the mechanism of NHE1-regulated cell survival, HRPT cells were pulsed with NH₄Cl to stimulate cytosolic acidification and immediate Na⁺/H⁺ exchange following NH₄Cl washout (19, 30–32). Inactive, cytosolic ERM requires phosphorylation at conserved carboxy-terminal Thr residues (ezrin, Thr⁵⁶⁷; radixin, Thr⁶⁴⁶, and moesin, Thr⁵⁵⁸) (33) to cause unfolding, translocation to the plasma membrane, and cross-linking between integral membrane proteins and cytoskeleton (34). Lysates from NH₄Cl-treated cells were assayed for the active ERM conformation by immunoblot analysis with anti-phospho-ERM antibodies. Fig. 3A (upper panel) shows increased ERM protein phosphorylation within 5 min, which is sustained for 2 h. Enhanced phospho-ERM content was not due to increased ERM expression, as evidenced by equivalent ezrin expression at all time points (Fig. 3A, lower panel).

Because NHE3 activity is also (a) associated with ERM binding (35), (b) abundant in proximal tubule in vivo, and (c) activated by NH₄Cl pulse, HRPT cells were screened for NHE3 expression by immunoblotting. These studies revealed undetectable HRPT cell NHE3 protein expression by immunoblot analysis (not shown), consistent with suppressed NHE3 expression in many proximal tubule cells lines.² Specificity of ERM regulation by NHE1 was further verified by hypertonic

² O. Moe, personal communication.
shrinkage, which activates NHE1 and inhibits NHE3 (26–28). The addition of 100 mM sucrose to cell culture medium induced peak ERM phosphorylation at 15 min, which remained sustained at 60 min (Fig. 3B).

Specificity of ERM phosphorylation following NHE1 activation was also assessed by comparing phospho-ERM content in NH4Cl-stimulated NHE-deficient PS120 fibroblasts versus PS120 cells transiently transfected to ectopically express NHE1. As expected, hypertonicity caused minimal ERM phosphorylation in PS120 cells (Fig. 3C, upper panel), whereas NHE1-transfected cells exhibited significantly greater basal and stimulated ERM phosphorylation, with a kinetic pattern similar to HRPT cells (Fig. 3C, lower panel). Furthermore, phospho-ERM content was significantly diminished in cells pretreated with 5 μM EIPA, suggesting that NHE1-dependent Na+/H+ exchange is linked to ERM activation. Collectively, the Fig. 3 data provide evidence that NHE1 activation stimulates ERM phosphorylation.

**NHE1 and ERM Directly Interact in the Cytoskeleton Fraction**—To determine whether NHE1-regulated ERM phosphorylation leads to cytoskeleton-based, physical NHE1-ERM interaction, NHE1 was stimulated with either NH4Cl (Fig. 4A) or sucrose (Fig. 4B) and then co-precipitated with phospho-ERM from Nonidet P-40-insoluble cytoskeleton fractions. Fig. 4 shows NHE1 interaction with phosphorylated ERM in the cytoskeleton-enriched compartment. NHE1-phospho-ERM binding was time-dependent, with peak interaction at 15 min, which was sustained for at least 60 min. The data represent the first evidence of direct association between NHE1 and phospho-ERM proteins.

**NHE1-Ezrin Interaction Protects against Apoptosis**—We have previously demonstrated that cells expressing a mutant NHE1 construct, with nonconserved Ala substitutions for membrane-proximal Lys and Arg residues (K/R/A), which impairs binding to negatively charged proteins, such as ERM (12), were more susceptible to apoptosis (11). To determine whether NHE1-dependent cell survival requires ERM interaction, LLC-PK1 cells stably transfected to express constitutively active and dominant negative ezrin were induced to undergo apoptosis by anisotonic shrinkage (Fig. 5A) or staurosporine exposure (Fig. 5B). Hypertonicity caused modest dose-dependent apoptosis in wild-type ezrin-expressing cells (Fig. 5A), consistent with previous results for hypertonicity-induced apoptosis in other kidney tubule cell lines (11). In cells expressing constitutively active ezrin (T567D), apoptosis was suppressed, whereas cells expressing dominant negative ezrin mutants (nonphosphorylatable point mutant (T567A), amino-terminal ezrin polypeptide, and Y353F point mutant that prevents PI3K binding) exhibited enhanced apoptosis (Fig. 5A). In cells expressing wild-type ezrin, staurosporine caused ~30% apoptosis (Fig. 5B), whereas cells expressing constitutively active ezrin (T567D) were relatively resistant to apoptosis, and dominant negative ezrin (T567A) expression accentuated apoptosis in response to staurosporine. Taken together, data from Figs. 4 and 5 demonstrate that activation and physical association of NHE1 and ezrin rescues cells from apoptosis, suggesting that NHE1-ERM interaction is an upstream step in the formation of a cell survival signalplex.

Because LLC-PK1 cells expressing Y353F ezrin mutants (Fig. 5A), which inhibit ERM-PI3K interaction, displayed increased susceptibility to apoptosis, the role of PI3K in LLC-PK1 cell apoptosis was specifically tested in cells pretreated with dissimilar PI3K inhibitors, wortmannin and LY294002. Fig. 5C demonstrates that like HRPT cells (11), untransfected LLC-PK1 cells were susceptible to staurosporine-induced apoptosis. Basal and staurosporine-stimulated apoptosis were enhanced by both PI3K inhibitors, consistent with a mechanism whereby PI3K promotes cell survival through interaction with the NHE1-ERM complex.

**Apoptosis Is Regulated by Akt Activity**—Although PI3K phosphorylates a variety of substrates, Akt is a well described effector molecule for PI3K and a key signaling enzyme in many cell survival pathways (36). To determine whether Akt regulates cell survival in our system, HRPT cells were pretreated with a chemical Akt inhibitor (Akt I) and then assessed for apoptosis. Fig. 6A reveals that Akt I accentuated staurosporine-induced apoptosis. Similar results were observed in cells induced to undergo apoptosis by hypertonic stimulation (not shown). To test for the role of Akt in cell survival by a different strategy, LLC-PK1 cells were pretreated with Akt siRNA, which resulted in significant suppression of Akt expression (Fig. 6B). Fig. 6C shows enhanced staurosporine-induced apoptosis in the Akt siRNA-treated group, indicating that Akt regulates cell survival. The data are consistent with a role for Akt as a downstream effector in a survival cascade that includes NHE1, ERM, and PI3K.

**NHE1 Stimulation Regulates Akt Activity**—To determine whether NHE1 activation is linked to Akt activity, NHE1 was stimulated by NH4Cl protocol, and cell lysates were probed for Akt activity, with anti-phospho-Akt antibodies. These experiments revealed time-dependent Akt phosphorylation, peaking after 30–60 min of NHE1 stimulation (Fig. 7A). The slower kinetics of Akt phosphorylation compared with ERM phosphorylation (Fig. 3A) suggests that Akt is downstream from ERM in the cell survival cascade. NHE1 activation by hypertonic sucrose stress also induced a 3-fold increase in phospho-Akt content (Fig. 7B). Anisotonic stress-induced Akt phosphorylation was diminished by selective NHE1 inhibition with EIPA (Fig. 7B), suggesting that NHE1-dependent Na+/H+ exchange also influences Akt activity.

**NHE1 Regulates Akt by an ERM-dependent Mechanism**—To establish that NHE1 and ERM proteins are linked to Akt activity, NHE1 was activated by NH4Cl pulse acidification in LLC-PK1 cell lines stably expressing wild-type and dominant negative ezrin constructs, and Akt activation was assessed by phospho-Akt immunoblot analysis. In wild-type, ezrin-transfected cells, Akt phosphorylation peaked at 30 min and remained increased at 60 min (Fig. 8A), consistent with data in HRPT cells (Fig. 7). Importantly, Akt was not activated by NHE1 stimulation in cells expressing dominant negative ezrin (Fig. 8A).

To confirm by a different approach that Akt is a downstream effector in an NHE1/ERM interaction-initiated cascade, LLC-PK1 cells rendered NHE-deficient were transiently transfected to express wild-type NHE1, the K/R/A NHE1 mutant that does not bind ERM proteins (12), or the Na+/H+ translocation-defective NHE1 point mutant, E2661 (12) (Fig. 8B); then stimulated with NH4Cl or sucrose; and probed for Akt phosphorylation by immunoblot analysis. NHE1 stimulation in wild-type
NHE1- or E266I-expressing cells resulted in increased phospho-Akt content (Fig. 8C). However, stimulation of KR/A NHE1 mutant did not cause Akt activation, thereby implicating ERM binding to activated NHE1 as a mechanism for Akt-dependent cell survival.

**DISCUSSION**

NHEs are expressed in all mammalian cells, and the ubiquitously expressed NHE1 isoform is most extensively characterized as a regulator of cell volume and pH. Because apoptotic cells undergo shrinkage and cytosol acidification, processes consistent with NHE1 inactivation, we have proposed that NHE1 is a cell survival factor. Our previous work has demonstrated that NHE1 expression and Na+/H+ exchange are associated with resistance to apoptosis (11). Cell survival in NHE-deficient cells was incompletely restored upon expression of NHE1-null cells are more sensitive to apoptosis compared with wild-type cells (11, 37), supporting a direct role for NHE1 in survival. The relative importance of NHE1 as a survival factor is established in the current studies by showing that ectopic NHE1 expression partially rescued cells induced to undergo apoptosis following cell survival.

NHE1 Recruits ERM for Cell Survival

**Fig. 5.** NHE1-ERM interaction protects against apoptosis. A, to determine whether NHE1 regulates ERM proteins and PI3K, NHE1 was stimulated with sucrose (6 h at 37 °C) addition to medium in LLC-PK1 cell lines stably transfected with wild-type ezrin, constitutively active (T567D) ezrin, amino-terminal ezrin (N-term) ezrin, or Y353F ezrin that prevents PI3K interaction. B, wild-type, T567D, and T676A cells were incubated with or without STS (5 μM for 6 h). *, p < 0.05 compared with wild-type, STS-treated cells by ANOVA.

**Fig. 6.** Apoptosis is regulated by Akt activity. A, HRPT cells were pretreated with Akt inhibitor (Akt I; 30 μM at 37 °C) for 1 h and then incubated with STS (5 μM for 6 h at 37 °C). The cells were stained with annexin V for apoptosis analysis. Similar results were observed in hypotonic sucrose-treated LLC-PK1 and HRPT cells (not shown). B, LLC-PK1 cells were transfected with 50 nM Akt siRNA or 50 nM integrin siRNA (control siRNA) for 48 h. The cells were then incubated with or without STS (5 μM for 6 h at 37 °C), and lysates were probed for Akt expression by immunoblot analysis. The blots were stripped and reprobed for α-tubulin as a control for protein loading and nonspecific RNA interference effects. C, LLC-PK1 cells were treated as described in B, but intact cells were instead assayed for apoptosis by annexin V labeling. Similar results were observed when apoptosis was determined by DAPI staining of chromatin (not shown).

**Fig. 7.** NHE1 stimulation regulates Akt activity. A, HRPT cell NHE1 was activated by NH4Cl pulse protocol. Whole cell lysates were harvested at indicated times and immunoblotted with anti-phospho-Akt antibodies (upper panel). The blots were stripped and reprobed with anti-Akt1 IgG (lower panel). B, HRPT cells were preincubated with EIPA (5 μM, 1 h, 37 °C), and then NHE1 was activated by sucrose (100 mM for 1 h at 37 °C) addition to medium. Whole cell lysates were probed for Akt activity by immunoblotting with anti-phospho-Akt antibodies (upper panel); the blots were stripped and reprobed with anti-Akt1 antibodies (lower panel) as a loading control. The histogram depicts densitometry data from three phospho-Akt blots, expressed as the means ± S.E. *, p < 0.05 compared with other groups by ANOVA.

NHE1 mutants with impaired ERM protein binding capacity, suggesting that NHE1-ERM interaction is required for cell survival (11). The current studies extend these findings by demonstrating that NHE1 is activated by apoptotic stress, which leads to direct interaction with ERM in the cytoskeleton-enriched cell fraction, and this interaction mediates recruitment of PI3K and Akt to regulate cell survival.

Previous studies demonstrate that NHE1-null cells are more sensitive to apoptosis compared with wild-type cells (11, 37), supporting a direct role for NHE1 in survival. The relative importance of NHE1 as a survival factor is established in the current studies by showing that ectopic NHE1 expression partially rescued cells induced to undergo apoptosis following cell survival.
lular K⁺ and Cl⁻ efflux. In contrast to previous conceptions that apoptosis-stimulated, intracellular K⁺ and Cl⁻ depletion irreversibly commit cells to die, we demonstrate that NHE1 activation prevents apoptosis in the context of concurrent robust ion efflux.

At least nine NHE isoforms have been identified, and all of the isoforms regulate electroneutral Na⁺ influx and H⁺ efflux, which could account for cell survival. NHE1 and NHE3 were the focus of our studies because both isoforms are expressed in kidney proximal tubule, from which cell lines for most of the experiments were derived, and both NHEs interact with ERM proteins (12, 35). Initial studies demonstrated transient intracellular alkalinization in response to apoptotic stress, consistent with either NHE1 or NHE3 activation. However, pH, changes were blunted by EIPA at concentrations that inhibit NHE1 but not NHE3 activity (26). Furthermore, apoptosis was rescued by ectopic NHE1 but not NHE3 expression, indicating that cell survival is NHE1-specific. Because NHE1 and NHE3 display divergent responses to anisotonic shrinkage, with cell volume reduction stimulating NHE1 and inhibiting NHE3 activity (26–28), selective rescue of apoptosis by NHE1 is in agreement with an NHE1-specific, cytoplasmic volume preservation mechanism of cell survival.

Cell survival signaling was initiated by interaction between NHE1 and ERM proteins. Although originally identified as cytoskeleton cross-linkers and important components of cell structure, ERM proteins are now recognized to also assemble and integrate actin-based signaling modules, with diverse downstream effects, that include adhesion, migration, secretion, and immunological synapse formation (16, 38). We speculate that NHE1 regulates cytoskeleton linkage to plasma membrane to maintain cell volume and shape, which facilitate proper enzyme-substrate targeting and spacing (39), as well as avoidance of molecular crowding, which has been implicated in osmotic shrinkage and spontaneous caspase activation (40, 41). In this regard, NHE1 interaction with ERM was specifically localized to cytoskeleton, indicating that compartmentalization is required for activation of appropriate downstream survival effectors. Studies with cells, either lacking NHE1, or with impaired NHE1-ERM interaction did not support cell survival, suggesting that signaling molecules were mislocated and/or excluded from the signaling complex. Cytoskeleton-based, NHE1-directed signaling mechanism is analogous to the focal adhesion complex, which clusters specific proteins, including NHE1 (12, 13, 42) to regions of cytoskeleton-plasma membrane contact. Because focal adhesion formation also promotes cell survival (43), we propose that NHE1 may be a key integrator for multiple, cooperative signaling cascades, which culminate in cell survival.

![NHE1 Recruits ERM for Cell Survival](image)

NHE1 Recruits ERM for Cell Survival

In the inactive state, ERM proteins reside within the cytosol in a closed conformation through head-to-tail interactions between the amino- and carboxyl-terminal domains. ERM proteins become activated following phosphorylation at conserved carboxyl-terminal Thr residues and interaction with negatively charged phosphatidylinositol 4,5-bisphosphate (PIP2), which causes protein unfolding and targeting to plasma membrane (33, 38). The mechanism of ERM activation in cell survival was not specifically investigated, but Rho kinase and protein kinase C have been shown to phosphorylate ERM (33, 38). In addition, the polybasic, membrane-proximal NHE1 domain that binds ERM, also binds PIP2 (44), suggesting that PIP2 could coordinately regulate assembly of an NHE1-ERM survival signaling complex. Although we cannot exclude that enhanced apoptosis in NHE1 KR/A mutant-expressing cells could be due to impaired interaction with negatively charged molecules, such as PIP2, rather than ERM, concordant findings in experiments with NHE1 KR/A and dominant negative ERM mutants support a role for ERM as an intermediary between NHE1 and cell survival signaling.

The hypothesis that NHE1-ERM interaction promotes cell...
survival was predicated upon the observation that ERM proteins directly bind to PI3K (14, 38). Multiple stimuli can activate PI3K, which then phosphorylates numerous substrates, thereby regulating cell survival, cytoskeletal rearrangement, and transformation (45). PI3K catalyzes phosphorylation of PI(3,4)P2 to PI(3,4,5)P3, which serves as a docking site for pleckstrin homology domain proteins, including Akt. Activated Akt facilitates cell growth, cell cycle entry, and cell migration, but the best described Akt function is mediation of cell survival, through phosphoregulation of multiple apoptosis-related proteins (13, 36). Interestingly, Akt has also been shown to regulate cell volume (45–47), suggesting that NHE1-dependent RVI and cell survival may be integrated through Akt signaling pathways. Our data indicate that NHE1-regulated ERM binding is critical for Akt activity, but the role of NHE1-dependent Na+/H+ exchange in Akt activation is less clear, because EIPA preincubation inhibited Akt phosphorylation (Fig. 7), whereas cell expressing the Na+/H+ -deficient E2661 NHE1 mutant maintained the capacity to activate Akt (Fig. 8). This discrepancy suggests that EIPA may exert effects beyond the inhibition of Na+/H+ translocation.

We previously demonstrated that NHE1 is a caspase substrate and that upon apoptotic cleavage, the loss of NHE1 function may have pathophysiologic consequences (11). Based upon our previous and current work, we propose that NHE1 is a critical cell survival factor, by mechanisms that involve Na+/H+ exchange to induce RVI and pH homeostasis, as well as through ERM protein binding that leads to sequential PI3K and Akt kinase activation (Fig. 9). NHE1 is commonly described as a housekeeping protein, which infers that it is merely a pedestrian molecule or a convenient control for biochemical experiments. However, many housekeeping tasks are required for normal cell function, and the current studies demonstrate that a vital NHE1 task is the regulation of cell survival.

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