Osmotic cell shrinkage activates ezrin/radixin/moesin (ERM) proteins: activation mechanisms and physiological implications

Maria Rasmussen,1 R. Todd Alexander,2 Barbara V. Darborg,1 Nadja Møbjerg,1 Else K. Hoffmann,1 András Kapus,3 and Stine F. Pedersen1

1Department of Molecular Biology, University of Copenhagen, Copenhagen, Denmark; and 2Cell Biology Program, Hospital for Sick Children, University of Toronto and 3St. Michaels Hospital Research Institute and Department of Surgery, University of Toronto, Toronto, Ontario, Canada

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Rasmussen M, Alexander RT, Darborg BV, Møbjerg N, Hoffmann EK, Kapus A, Pedersen SF. Osmotic cell shrinkage activates ezrin/radixin/moesin (ERM) proteins: activation mechanisms and physiological implications. Am J Physiol Cell Physiol 294: C197–C212, 2008. First published October 31, 2007; doi:10.1152/ajpcell.00268.2007.—Hyperosmotic shrinkage induces multiple cellular responses, including activation of volume-regulatory ion transport, cytoskeletal reorganization, and cell death. Here we investigated the possible roles of ezrin/radixin/moesin (ERM) proteins in these events. Osmotic shrinkage of Ehrlich Lettre ascites cells elicited the formation of long microvillus-like protrusions, rapid translocation of endogenous ERM proteins and green fluorescent protein-tagged ezrin to the cortical region including these protrusions, and Thr567/564/558 (ezrin/radixin/moesin) phosphorylation of cortical ERM proteins. Reduced cell volume appeared to be the critical parameter in hypertonicity-induced ERM protein activation, whereas alterations in extracellular ionic strength or intracellular pH were not involved. A shrinkage-induced increase in the level of membrane-associated phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] appeared to play an important role in ERM protein activation, which was prevented after PtdIns(4,5)P2 depletion by expression of the synaptojanin-2 phosphatase domain. While expression of constitutively active RhoA increased basal ERM protein phosphorylation, which was also unaffected by the inhibition or absence of Na+/H+ exchanger isoform (NHE1). Ezrin knockdown by small interfering RNA increased shrinkage-induced NHE1 activity, reduced basal and shrinkage-induced Rho activity, and attenuated the shrinkage-induced formation of microvillus-like protrusions. Hyperosmolarity-induced cell death was unaltered by ezrin knockdown or after phosphatidylinositol 3-kinase (PI3K) inhibition. In conclusion, ERM proteins are activated by osmotic shrinkage in a PtdIns(4,5)P2-dependent manner. This in turn mitigates the shrinkage-induced activation of NHE1, augments Rho activity, and may also contribute to F-actin rearrangement. In contrast, no evidence was found for the involvement of an NHE1-ezrin-PI3K-PKB pathway in counteracting shrinkage-induced cell death.

RhoA; Na+/H+ exchanger 1; cell volume; cytoskeleton; phosphatidylinositol 4,5-bisphosphate

THE EZRIN/RADIXIN/MOESIN (ERM) protein family cross-links plasma membrane proteins with F-actin and plays a major role in the control of cell morphology, migration, and adhesion and a variety of cellular signaling processes (7, 23). Notably, recent evidence has assigned important roles for ERM proteins in cancer development and metastasis (25). ERM proteins and the closely related protein merlin (the product of the Neurofibromatosis 2 gene) belong to the Band 4.1 superfamily, members of which contain a highly conserved NH2-terminal FERM (four-point-one, ezrin/radixin/moesin) domain (7). ERM proteins are maintained in an inactive cytosolic state by intramolecular association between the FERM domain (also called the NH2-terminal ERM association domain, or N-ERMAD) and a COOH-terminal region, the C-ERMAD (7, 23). In this closed conformation, the COOH-terminal F-actin binding site as well as the NH2-terminal binding sites for at least some of the ligands are masked (7). In their activated state, ERM proteins are able to associate with F-actin through a COOH-terminal site and with integral membrane proteins (either directly or indirectly, e.g., via the EBPs family of scaffolding proteins) through the FERM domain. Two events appear to be required for ERM protein activation: interaction of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] with the FERM domain and phosphorylation of Thr567 (ezrin)/Thr564 (radixin)/Thr558 (moesin) in the COOH-terminal region (7, 23). It is thought that PtdIns(4,5)P2 is required for the activation and translocation of ERM proteins to the membrane, while phosphorylation is required for maintaining the active state (56).

Several Ser/Thr protein kinases, including protein kinase C (PKC)-α, PKC-δ, myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), G protein-coupled receptor kinase 2 (GKR2), and the ste20-related kinase Nck-interacting kinase (NIK) have been proposed to mediate the threonine phosphorylation of ERM protein in vitro and/or in vivo (3, 9, 23). Rho kinase also mediates ERM protein phosphorylation in vitro; however, it is controversial whether this plays a role in vivo. Thus, although Rho activation has been shown to induce ERM protein activation in vivo, this has been proposed to reflect the involvement of another Rho effector, the phosphatidylinositol 4-phosphate 5-kinase (PI4P5K). Rho-mediated activation of PI4P5K might elevate the intracellular level of PtdIns(4,5)P2 (32, 38), which in turn would stabilize the ERM proteins in an open conformation, thereby permitting phosphorylation at the crucial Thr residue. Importantly, the converse relation has also been suggested: Rho has been found to be regulated by ERM proteins as a result of an inhibitory interaction between ERM proteins and Rho-GDP-dissociation inhibitor (Rho-GDI) (49).

Once activated, ERM proteins play important roles in control of cytoskeletal organization, and hence cell morphology, adhesion, and migration (Refs. 15, 16, 50; see Ref. 7). In
particular, a major role for ERM proteins in formation of microvilli, filopodia, lamellipodia, microspikes, and other F-actin-containing protrusions has been demonstrated in a wide range of cell types (Refs. 3, 27, 45, 46, 50; see Ref. 7). ERM proteins are also implicated in the control of cell death/survival balance (18, 51) and in regulation of the localization and activity of membrane transport proteins (19, 57).

Osmotic cell shrinkage, which occurs under physiological and pathophysiological conditions in multiple cell types, elicits a wide range of cellular responses, including rapid activation of RhoA (10), increases in cellular PtdIns(4,5)P2 levels (34, 53, 21), and extensive cytoskeletal rearrangement (11, 42), all events consistent with activation of ERM proteins. Also pointing to a possible link between ERM proteins and cellular responses to shrinkage, ezrin associates directly with the ubiquitous, shrinkage-activated plasma membrane Na+ /H+ exchanger, NHE1 (15, 16). Direct interaction of the COOH-terminal cytoplasmic tail of NHE1 with ezrin has been shown to play a central role in cell migration and organization of the cortical cytoskeleton (15, 16). Osmotic shrinkage eventually leads to cell cycle arrest and programmed cell death (see Refs. 2, 11, 22), and it was recently suggested that ezrin recruitment to NHE1 is involved in the regulation of cell survival after osmotic stress and other apoptotic stimuli via recruitment of phosphatidylinositol 3-kinase (PI3K) and consequent activation of protein kinase B (PKB) (51). In conjunction with the plasma membrane-cytoskeleton linker function of ERM proteins, which renders them attractive candidates for sensing of cell volume perturbations, this led us to hypothesize that ERM proteins might be activated by osmotic stress and act as upstream regulators of the cellular response to osmotic shrinkage. Given that the interaction with NHE1 has been shown in vivo only for ezrin (16), and that the interaction with PI3K appears to be ezrin specific (see Ref. 23), we focused in particular on the role of ezrin.

Here we tested the hypothesis that activation of ERM proteins, ezrin in particular, might be an early signal of osmotic cell perturbation and investigated the mechanisms responsible for, and the physiological consequences of, ERM protein activation induced by cell shrinkage. We report that osmotic cell shrinkage elicits rapid plasma membrane translocation and activation of ERM proteins in a manner dependent on PtdIns(4,5)P2 and independent of NHE1. In Ehrlich Lette ascites (ELA) cells, the shrinkage-induced stimulation of ezrin activity in turn reduces NHE1 activity, contributes to the hypertonicity-triggered Rho activation, and may participate in F-actin rearrangement, while it does not regulate shrinkage-induced cell death.

Some of these findings have been published previously in abstract form (14).

EXPERIMENTAL PROCEDURES

Reagents and Solutions

Reagents were of the highest analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO) or Mallinckrodt Baker (Deventer, The Netherlands) unless otherwise stated. 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and 2',7'-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein, tetra-acetoxyethyl ester (BCECF-AM) were from Molecular Probes (Leiden, The Netherlands), and were dissolved at 2.5 mM in double-distilled (dd)H2O and at 1.2 mM in desiccated DMSO. Nigericin (Sigma) was dissolved at 5 mM in EtOH. Y-27632 was from Calbiochem (Darmstadt, Germany) and was dissolved at 5 mM in ddH2O, and wortmannin (Sigma-Aldrich) was dissolved at 1 mM in DMSO. PDGF-BB was also from Calbiochem and was dissolved at 100 μM in dilute HCl. All these stock solutions were stored at −20°C. Stock solutions of paraformaldehyde (20% wt/vol in ddH2O) were prepared fresh regularly and stored at 4°C. Monoclonal anti-ezrin antibody was from Sigma, and antibodies recognizing total ERM, and ezrin, radixin, and moesin phosphorylated at Thr567, Thr642, and Thr558, respectively (corresponding to the active state of these proteins), and extracellular signal-regulated kinase (ERK)1/2 phosphorylated at Thr202 and Tyr204 were from Cell Signaling (Beverly, MA), as was the antibody against PKB phosphorylated at Ser473.

The standard Ringer solution for ELA cells contained (in mM) 143 NaCl, 5 KCl, 1 MgSO4, 1 Na2HPO4, 1 CaCl2, 3.3 MOPS, 3.3TES, and 5 HEPEs (pH 7.4, 310 mosM). The standard hypertonie (600 mosM) Ringer solution was prepared by doubling the concentrations of all components except MOPS, TES, and HEPEs compared with the standard medium or, where indicated, by addition of mannitol to the isotonic medium. In HCO3– medium, 25 mM NaCl was replaced by 25 mM NaHCO3. In the KCl medium used for calibration of BCECF fluorescence, KCl was substituted for NaCl in equimolar amounts.

Cells and Cell Culture

All cell cultures were maintained at 37°C and 5% CO2 in a humidified incubator. Cells were passaged every 3–4 days, and only passages 6–30 were used. All media were supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution (except for medium for COS-7 cells, which did not contain penicillin-streptomycin). The cell cultures and culture media used were ELA cells [American Type Culture Collection (ATCC), Manassas, VA] maintained in RPMI-1640 medium (Sigma); wild-type NIH3T3 cells (clone 7); a kind gift from Prof. B. Willumsen (Institute of Molecular Biology, University of Copenhagen), and NIH3T3 cells stably expressing constitutively active Rac1 and RhoA, prepared as previously described (40) and maintained in α-modified Eagle’s medium (DMEM); and AP1 cells and S5 cells [AP1 cells stably expressing human NHE1, a kind gift from Prof. P. M. Cala (School of Medicine, University of California, Davis) maintained in α-modified Eagle’s medium with 1% l-glutamine and, for transfected cells, 400 μg/ml G418-sulfate (GIBCO BRL, Invitrogen, Carlsbad, CA). Finally, COS-7 cells were obtained from ATCC and maintained in DMEM. The porcine proximal tubular cell line LLC-PK1 was cultured in DMEM medium and grown to full confluence before experimentation. Unless otherwise noted, experiments were carried out at 37°C.

Constructs and Transient Transfection

The plasmid containing ezrin-green fluorescent protein (GFP) was a gift of Dr. Antony Bretscher (Cornell University, Ithaca, NY). The plasmid containing the phosphatase domain (PD) of synaptojanin 2 was a gift of Dr. Marc Symons (Feinstein Institute for Medical Research, Manhasset, NY), and (31), and plasmids containing mRFP-PLC and mRFP-H-Ras were gifts of Dr. S. Grinstein (University of Toronto, Ontario, Canada) (54). The above constructs were expressed in ELA cells with 2 μg of DNA and Lipofectamine 2000, following the manufacturer’s instructions. Experiments were performed 24–48 h after transfection. Myc-tagged constitutively active Rho (RhoQ63L) (10) was expressed in ELA cells grown in 10-cm petri dishes, with Lipofectamine 2000 and 4 μg of DNA per dish, and these cells were used for experiments 48 h after transfection.

Small Interfering RNA-Mediated Knockdown of Ezrin

Desalted, annealed, 21-bp small interfering RNA (siRNA) duplexes with 2 nt overhangs were obtained from Dharmacon RNA Technologies (Lafayette, CO). The target sequence for ezrin was 5’-CAAGAAGGCACCUGACUUU-3’, corresponding to position
872–890 in mouse ezrin. A %GC-matched scrambled 21-bp oligomer, also from Dharmacon, was used as a control. Cells were transfected with ezrin- or control siRNA at 100 nM with Lipofectamine 2000. After 72 h cells were harvested and knockdown was confirmed by immunoblotting for ezrin, or the cells were used in experiments as indicated.

**RNA Isolation, Reverse Transcription, and PCR**

Total RNA was isolated by standard procedures and reverse transcribed with Superscript II reverse transcriptase and random primers (Invitrogen). PCR was carried out with Taq DNA polymerase (Invitrogen) and the following protocol: 95°C for 2 min, 35 × (95°C for 30 s, 40°C for 30 s, 72°C for 2 min), 72°C for 10 min. Primer sequences used were: ezrin forward 5′-ACAGCAGTTGAAACCGG-3′, reverse 5′-GGCTCCAGACGTTCCAGG-3′ (expected product size 213 bp); radixin: forward 5′-TCTCCTCATTTTATGAAAAGA-3′, reverse 5′-GCAGCAGTGCAGAATCTC-3′ (expected product size 370 bp); moesin: forward 5′-TGCTCTCTGGAAATGAGA-3′, reverse 5′-CAGAAGCTGGAGAGA-3′ (expected product size 263 bp); merlin: forward 5′-GAAGGAGGTATGAGCTGC-3′, reverse 5′-TCCTGAGCTTGC-3′ (expected product size 460 bp). The specificity of the reactions was confirmed by control reactions in which primers or cDNA were replaced by ddH2O. PCR products were separated on 0.8% agarose gels with kb markers (Invitrogen) and visualized with ethidium bromide.

**Gel Electrophoresis and Western Blotting**

Cells were lysed in boiling lysis buffer (1% SDS, 10 mM Tris·HCl, pH 7.5), homogenized, and debris cleared by centrifugation. After protein determination (bicinchoninic acid protein kit, Bio-Rad, Hercules, CA), equal amounts of protein per well were diluted in NuPage LDS sample buffer (Invitrogen) containing 29% DTT, boiled for 5 min, subjected to 10% SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were stained with Ponceau Red, washed, blocked in Tris-buffered saline (TBS) + Tween (TBST; 0.01 M Tris·HCl pH 7.4, 0.15 M NaCl, and 1% Tween 20) containing 5% nonfat dry milk, incubated with primary antibody in blocking buffer, and washed in TBST. Antibody binding was visualized by incubation with the relevant alkaline phosphatase-conjugated secondary antibody, wash in TBST, and detection with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Membranes were scanned, and band intensity was estimated from arbitrary densitometric values obtained with UN-SCAN-IT software. The total level of ERM, i.e., the sum of the 80-kDa band (corresponding to ezrin and radixin) and the ~75-kDa band (corresponding to moesin), and phospho-ERM protein was obtained. Unless otherwise indicated, the phospho-ERM level was normalized to the total current ERM level and taken relative to the isotypic value.

**Immunocytochemistry and Confocal Laser Scanning Microscopy**

Cells grown on HCl- and EtOH-washed glass coverslips were washed in isotonic Ringer solution and allowed to equilibrate in this solution for 20–30 min (37°C). After stimulation as indicated, cells were fixed in 2% paraformaldehyde (15 min at room temperature followed by 30 min on ice), washed in TBS (in mM: 150 NaCl, 10 Tris·HCl, 1 MgCl2, 1 EGTA, pH 7.3), permeabilized for 10 min (0.2% Triton X-100 in TBS), blocked for 30 min (5% BSA in TBS), incubated with primary antibodies against either total or phosphorylated ERM proteins (1:100 in TBS overnight, 4°C), washed extensively in TBS, and incubated with FITC-conjugated goat anti-mouse antibody (1:600 in TBS, 1 h) in the presence or absence of 2 U/ml rhodamine-conjugated phalloidin to label F-actin. Finally, coverslips were washed extensively in TBS and mounted with x-propyl gallate 2% (wt/vol) in PBS-glycerin. Fluorescence was visualized with a ×40 1.25-numerical aperture (NA) or a ×100 1.4-NA plan apochromat objective and the 488- and 568-nm laser lines of a Leica DM IRBE microscope with a Leica TSC NT confocal laser scanning unit (Leica Lasertechnik, Heidelberg, Germany). Optical slice thickness was 1 μm, and pinhole size was 1 airy disk. Images shown are frame averaged and presented in RGB pseudocolor. No or only negligible labeling was detectable in the absence of primary antibody. Quantification of cortex-to-cytoplasm ratios of endogenous ezrin was performed by defining regions of interest (ROIs) in the cytoplasmic and cortical regions (the latter identified from colabeling for cortical F-actin). The average pixel intensity per ROI was determined, and data were normalized to cytosolic values.

**Scanning Electron Microscopy**

Scanning electron microscopy (SEM) was employed for high-resolution imaging of ELA cell surfaces. ELA cells were seeded on HCl- and EtOH-cleaned 5 × 5-mm glass coverslips 3 days before fixation. The cells were exposed to either iso- or hypertonic (60 mosM) Ringer solution for 5 min, washed twice in ice-cold PBS, and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 120 min at 4°C. The cells were then washed twice in 0.1 M cacodylate buffer for 30 min and postfixed in 1% OsO4 in 0.1 M cacodylate buffer for 90 min at 4°C. After postfixation the cells were dehydrated through a graded ethanol series (25%, 50%, 70%, 80%, 96%, and 99% for 10 min per step at room temperature and left in 99% for 30–60 min) and transferred to acetone through a graded series of EtOH and acetone. Subsequently, the cells were critical point dried in liquid carbon dioxide with a BAL-TEC CPD 030 critical point dryer. The coverslips were mounted on aluminum stubs, and the cells were sputter-coated with platinum-palladium for 60 s with a JEOL JFC-2300 HR. ELA cells were examined in a JEOL JSM-6335-F field emission scanning electron microscope.

**Live Cell Imaging of Ezrin-GFP**

ELA cells were grown on 25-mm glass coverslips and transfected with the indicated constructs using Lipofectamine 2000, as per the manufacturer’s protocol. Twenty-four hours after transfection, cells were transferred to the stage of a Yokogawa spinning-disk-inverted fluorescence microscope, and image acquisition was performed with Volocity V3.6 (Improvision) software. After imaging under isotonic conditions, the cells were exposed to hypertonic medium and imaged at the times indicated. Quantification of cortex-to-cytoplasm ratios of GFP-ezrin was performed as above, except that the cortical region was identified from coexpression of mRF-H-Ras (Fig. 3) or mRF-PH-PLC (Fig. 6). The average pixel intensity per ROI was determined, and data were normalized to cytosolic values.

**Fluorescence Recovery After Photobleaching**

Experiments were performed and analyzed as described previously (1). In brief, ELA cells were transfected with ezrin-GFP and samples placed in Attofluor chambers were mounted on the stage of a confocal laser microscope (Zeiss LSM 510) and bathed in either isosmolar or hyperosmolar solution. A juxtamembrane region was brought into focus, and two equal areas (2-μm diameter) were defined. Five minutes after bathing in either isosmolar or hyperosmolar solution two baseline fluorescence measurements were acquired; one of the selected areas was then irreversibly photobleached, and the fluorescence of both areas was measured over time. The fractional fluorescence recovery of the bleached area was determined relative to the average of the two prebleach measurements. The unbleached area was used to estimate possible bleaching incurred during image acquisition. Fluorescence recovery after photobleaching (FRAP) data were fitted by nonlinear regression analysis with the equations by Yguerabide et al. (55).
**Rho Activity Measurements**

Cellular Rho-GTP levels were estimated with a commercial assay (Cytoskeleton, Denver, CO). Cells were grown in 10-cm petri dishes and in some cases transfected with ezrin siRNA 72 h before experiments as indicated above. After exposure to iso- or hypertonic Ringer solution for the time indicated, cells were quickly washed in PBS, lysed in 500 μl of lysis buffer containing protease inhibitors, scraped off, and cleared by centrifugation (20,000 g for 5 min at 4°C). Five hundred microliters of cleared lysate was transferred to 20 μl of RhoA-binding domain (RBD) bead solution, 5 μl of protease inhibitor cocktail was added, and lysate and beads were incubated at constant rotation for 1 h at 4°C. The beads were washed once in lysis binding buffer and once in washing buffer (5,000 g, 3 min, 4°C). RBD beads with bound Rho-GTP were spun down as above, the supernatant was carefully removed, the RBD bead pellet was dissolved in 10 μl of nigericin/high-K+ rich medium titrated to pH 7.0 and cleared by centrifugation (20,000 g for 5 min at 4°C). Five hundred microliters of cleared lysate was transferred to 20 μl of nigericin/high-K+ rich medium and incubated at 37°C for 10 min. Extracellular dye was washed away, and Na+/H+ exchange was initiated with addition of NaCl to the RPMI medium from a sterile 3 M stock solution to a final concentration of 150 mM, resulting in a total osmolality of 600 mosM compared with 300 mosM in isotonic RPMI medium. Data from a single experiment are the means of values from six identical wells for each condition. Viability was calculated as the absorbance relative to that under isotonic control conditions after background subtraction (wells containing the relevant medium but no cells).

**Estimation of Na+/H+ Exchanger Activity**

Shrinkage-induced NHE1 activity was assessed as the hypertonicity-induced intracellular pH (pHi) increase in nominally HCO3−-free medium, which in ELA and COS-7 cells reflects NHE1 exclusively. Dual excitation ratio determination of BCECF fluorescence was used to measure pHi, as previously detailed for ELA (39) and COS-7 (24) cells.

ELA cells. Briefly, cells grown to confluence on rectangular HCl- and ethanol-cleaned glass coverslips were incubated for 30 min at 37°C with 1.2 μM BCECF-AM in the standard isotonic solution, washed, and further incubated for 15 min in this solution. The cells were mounted in the perfused cuvette of a PTI Ratiomaster spectrophotometer, and emission was detected at 525 nm after excitation at 445 nm and 495 nm. The 445 nm-to-495 nm ratio was calculated after subtraction of background fluorescence (unloaded cells in the relevant experimental solution), and calibration to pHi was performed with a seven-point nigericin/high-K+ calibration (based on Ref. 6). Because the initial pHi was very similar in all experiments, the rate of change in pHi after hypertonic challenge was calculated as the slope of the initial linear part of the curve (0 to 2–3 min after hypertonic challenge).

COS-7 cells. Briefly, cells grown on 25-mm glass coverslips and transfected with siRNA as indicated above were placed in Attofluor cell chambers, mounted on the microscope stage, and loaded with 5 μg/ml BCECF-AM in isotonic medium at 37°C for 10 min. Extracellular dye was washed away, and Na+/H+ exchange was initiated by introduction of hypertonic solution. Calibration to pHi was performed by equilibrating the cells with K+-rich medium titrated to defined pH values and containing 10 μg/ml nigericin.

**Quantification of Cellular F-Actin Levels**

Net F-actin content was estimated with a quantitative rhodamine-phalloidin binding assay essentially as described previously (43). Cells seeded in six-well dishes to a density of 100,000 cells per well at the time of the experiment were fixed in 2% paraformaldehyde (15 min at room temperature, 30 min on ice), followed by three washes in TBS, permeabilization for 10 min in saponin buffer, incubation with 10 U/ml rhodamine-phalloidin in saponin buffer (MOPS buffer + 0.1% saponin) for 1 h, and three washes in MOPS buffer (in mM: 5 MOPS, 5 EGTA, 20 K2HPO4, 2 MgSO4, pH 6.9). Rhodamine label was extracted in gradient-grade methanol per well by gentle agitation for 30 min, and rhodamine fluorescence was measured spectrophotometrically (excitation 540 nm, emission 576 nm). The assay was linear in the relevant range, and specificity for F-actin was verified by competition with a 100-fold excess of unlabeled phalloidin. Data are shown as the 576-nm emission intensity after subtraction of a methyl blue blank, relative to the corresponding isotonic control.

**Estimation of Cell Viability**

Cells were seeded in 96-well plates to reach 30,000 cells per well on the day of the experiment. Cell viability was estimated from the fraction of functional mitochondria with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (37). Hypertonic medium was prepared by addition of NaCl to the RPMI medium from a sterile 3 M stock solution to a final concentration of 150 mM, resulting in a total osmolality of 600 mosM compared with 300 mosM in isotonic RPMI medium. Data from a single experiment are the means of values from six identical wells for each condition. Viability was calculated as the absorbance relative to that under isotonic control conditions after background subtraction (wells containing the relevant medium but no cells).

**Data Analysis and Statistics**

Data are presented as standard error of the mean (means ± SE) of at least three independent experiments or as individual experiments representative of at least three independent experiments, unless otherwise indicated. Double-sided Student’s t-test was used for statistical evaluation, with 0.05 as the level of significance.

**RESULTS**

**Effect of Hyperosmotic Stress on ERM Proteins**

Immunolabeling of ELA cells with an antibody recognizing ezrin, radixin, and moesin (ERM) showed that under isotonic conditions, ERM proteins were located throughout the cytoplasm and cortical region (Fig. 1A). Phosphorylated ERM proteins were also detectable under isotonic conditions, predominantly in the cortical region. Osmotic cell shrinkage induced by hypertonic saline caused a significant increase in ERM protein phosphorylation already after 1 min, indicating that ERM proteins are rapidly activated by osmotic cell shrinkage. Increased ERM phosphorylation was most pronounced in the cortical regions, although some enhancement was also seen in the cytosol (Fig. 1A). To ascertain that the increased cortical phospho-ERM fluorescence was not an artifact of shrinkage, we also tested the effect of shrinkage on the localization of phosphorylated ERK1/2 as a control. Phospho-ERK1/2 exhibited a completely different relocalization pattern, from an approximately equal nuclear-cytosolic distribution under isotonic conditions to a largely cortical cytoplasmic distribution under hypertonic conditions, as we previously reported (39), with no detectable translocation to the cortical region (Fig. 1A, e, f). As seen in Fig. 1B, osmotic shrinkage greatly increased the density, and apparently also the length, of microvillus-like protrusions. F-actin and phosphorylated ERM proteins colocalized in these protrusions, although the phosphorylated ERM proteins tended to be found most predominantly in the more distal part and tip and were often missing from the base. In contrast, there was no detectable colocalization of phospho-ERM with stress fibers (Fig. 1B, g, inset).

At the mRNA level, ELA cells express all three ERM proteins (ezrin, radixin, moesin), as well as the related protein merlin (data not shown; n = 3 independent mRNA prepara-
Fig. 1. Localization and phosphorylation of ezrin/radixin/moesin (ERM) proteins in Ehrlich Lettre ascites (ELA) cells under iso- and hypertonic conditions. 

A: ELA cells grown on 22-mm glass coverslips were exposed to isotonic (300 mosM, 10 min) or hypertonic (600 mosM, 1 min) Ringer solution, paraformaldehyde fixed, permeabilized, blocked, and labeled with primary antibodies against either total (a, b) or Thr567/Thr564/Thr558-phosphorylated (c, d) ERM proteins and the relevant FITC-conjugated secondary antibodies. Fluorescence was analyzed by confocal laser scanning microscopy using a ×40 1.25-numerical aperture objective, and the images are 1 μm optical sections taken at a distance from the coverslip of 1/3 total cell height, at identical magnification (digital zoom 2.5). Images shown are representative of 3–4 (total ERM) or 5–7 (p-ERM) independent experiments for each condition. As a control (ctrl) for shrinkage artifacts, the distribution of extracellular signal-regulated kinase (ERK)1/2 phosphorylated at Thr202 and Tyr204 under isotonic (e) and hypertonic (f) (15 min) conditions is shown. As seen, phosphorylated ERK translocates from the nucleus to the cytosol, but not to the cortical region, on osmotic shrinkage (n = 3). Essentially no labeling was detectable in the absence of primary antibody (g).

B: cells were exposed to isotonic (a–d, 300 mosM, 10 min) or hypertonic (e–h, 600 mosM, 5 min) Ringer solution. For immunofluorescence cells were fixed, labeled, and visualized as described in A, except that rhodamine-conjugated phallodin (2 U/ml) was included with the secondary antibody to label F-actin. Inset in g shows an optical slice taken close to the bottom of the cell, illustrating the apparent lack of colocalization of phospho-ERM proteins with stress fibers. Images shown are representative of 4 or 5 independent experiments for each condition. Essentially no labeling was detectable in the absence of primary antibody (not shown). For scanning electron micrographs (d, h) cells were fixed, dehydrated, and coated as described in EXPERIMENTAL PROCEDURES and visualized with a JEOL JSM-6335-F field emission scanning electron microscope (SEM). SEM images shown are representative of 3 independent experiments for each condition.
tions and PCR analyses for each), in agreement with previous reports that these proteins are often coexpressed in cultured cells (28). We were specifically interested in ezrin, since this is the only ERM protein that has been shown previously to associate with NHE1 in vivo (15, 16) and the only isoform implicated in PI3K recruitment (23). Moreover, ezrin has been found to regulate another volume-sensitive NHE isoform, NHE3 (57), and to modulate RhoA activity (49), which is also volume sensitive (48).

Endogenous ezrin exhibited a mostly cytosolic localization pattern in ELA cells under isotonic conditions and a partial translocation toward the cortical region on osmotic shrinkage in the great majority of cells studied (Fig. 2A). This pattern was confirmed by transfection of ELA cells with GFP-ezrin (Fig. 2B). For unequivocal localization of the plasma membrane for the quantification of translocation, the cells in Fig. 2B were cotransfected with the red fluorescent protein (RFP)-tagged tail of H-Ras (54), which associates with the plasma membrane via two palmitoyl anchors. As seen, under isotonic conditions GFP-ezrin was evenly distributed in cytosol, and on cell shrinkage a partial translocation to the cortical region and microvillus-like protrusions was evident.

FRAP has been used previously to distinguish between the cytosolic and membrane-associated forms of ezrin (13). FRAP experiments in cells transfected with GFP-ezrin demonstrated a substantial reduction in ezrin mobility in the shrunk cells (Fig. 2C). As seen, the total fluorescence recovery was greatly reduced after 5 min of osmotic shrinkage (from 93.1% ± 3.6% under isotonic conditions to 63.7% ± 3.1% under hypertonic conditions; n = 10 for each condition). This indicates that a substantial fraction of ezrin becomes fully immobilized under hypertonic conditions, consistent with the notion that the association of ezrin with plasma membrane/cytoskeletal structures was increased by osmotic shrinkage. Moreover, the halftime to reach the plateau phase was substantially increased by osmotic shrinkage (from 3.7 ± 1.06 s under isotonic conditions to 15.8 ± 3.33 s under hypertonic conditions), suggesting that the mobility of the remaining fraction was also reduced (see DISCUSSION).

Jointly, these findings demonstrate that ERM proteins, and specifically ezrin, are rapidly phosphorylated and translocated to the cell periphery on osmotic cell shrinkage.

Mechanisms of Shrinkage-Induced ERM Protein Activation

Lack of dependence on HCO3- concentration or extracellular ionic strength. In Figs. 1 and 2, osmotic cell shrinkage was induced by increasing the extracellular salt concentration in the nominal absence of HCO3-. Under these conditions, the shrinkage-induced NHE1 activity results in a robust intracellular alkalinization, which is abolished or greatly attenuated in the presence of HCO3- (Ref. 39; see Ref. 41). Moreover, some cellular responses to osmotic stress are dependent on whether or not extracellular ionic strength is increased (see, e.g., Ref. 44). We therefore considered the effects of HCO3- and ionic strength on shrinkage-induced ERM protein phosphorylation. To allow direct quantitative evaluation, and to verify the immunofluorescence analysis with an alternative method, these experiments were performed by Western blotting. Confirming the immunofluorescence data, osmotic shrinkage was associated with increased ERM protein phosphorylation (Fig. 3). As seen, the increase in phosphorylation was similar whether extracellular osmolarity was increased in the absence or presence of 25 mM HCO3-, arguing against the involvement of changes in pH, in shrinkage-induced ERM protein phosphorylation. The increase in ERM protein phosphorylation was slightly greater when cells were shrunk at unaltered extracellular ionic strength (mannitol). It may also be noted that, consistent with the PCR data showing the presence of several ERM proteins in ELA cells, two bands are labeled by the total ERM protein antibody, corresponding to ezrin and radixin (~80 kDa) and moesin (~75 kDa), respectively, and two phospho-ERM bands are also detectable. Rapid, shrinkage-induced ERM protein phosphorylation was also detected in LLC-PK1 kidney tubular epithelial cells (n = 3; data not shown), in COS-7 cells (n = 3; data not shown), and in NIH3T3 and AP1 cells (see below), indicating that it occurs in a wide variety of cell types.

Lack of involvement of the Na+/H+ exchanger NHE1. NHE1 is rapidly activated by osmotic shrinkage of ELA cells, and its activation is completely abolished in the presence of the NHE1 inhibitor EIPA at 5 μM (39). In light of the recent report that ERM proteins are phosphorylated and recruited to NHE1 in a manner inhibited by EIPA (51), we assessed whether the shrinkage-induced ERM protein phosphorylation in ELA cells was dependent on NHE1 activity. Confirming the immunofluorescence data, Western blotting demonstrated that the relative ERM protein phosphorylation level increased during the first 30 min after hypertonic exposure (Fig. 4A). Inhibition of NHE1 activity by EIPA did not affect the shrinkage-induced increase in ERM protein phosphorylation (Fig. 4A), indicating that this phenomenon is independent of the transport activity of NHE1. Because ERM proteins can directly associate with NHE1 (16), we also considered the possibility that the presence of NHE1, rather than its activity, might be required for the shrinkage-induced effect on ERM proteins. However, shrinkage-induced ERM protein phosphorylation was similar in AP1 cells, which

Fig. 2. Localization and shrinkage-induced translocation of ezrin in ELA cells. A: cells were treated and imaged as described in Fig. 1, except that the antibody used was a monoclonal ezrin antibody. Images shown illustrate cells under isotonic conditions (top) and after 5 min of hypertonic exposure (bottom). a–d: Ezrin. e–f: F-actin (rhodamine phalloidin). g: Quantification of cytoplasmic and cortical ezrin labeling. Images shown are representative of 3 independent experiments for each condition, taken as described for Fig. 1, at identical magnification (digital zoom 2). Quantification was performed by defining regions of interest (ROIs) in the cytoplasmic and cortical regions (the latter identified from colabeling for cortical F-actin labeling). The average pixel intensity per ROI was determined, and data were normalized to the cytosolic values and given as means with SE error bars. B: cells were cotransfected with green fluorescent protein (GFP)-tagged ezrin and red fluorescent protein (mRFP)-H-Ras as described in EXPERIMENTAL PROCEDURES, exposed to hypertonic conditions for 5 min, and monitored by spinning-disk confocal microscopy. a: Ezrin. b: F-actin (rhodamine phalloidin). c: mRFP-H-Ras. d: Merge. e: Quantification of cytoplasmic and cortical GFP-ezrin. Experiment shown is representative of 12 independent experiments. Quantification of cytoplasmic and cortical GFP-ezrin was performed as in A, except that the plasma membrane (PM) was identified from the colabeling for mRFP-H-Ras. C: cells were transfected with GFP-ezrin and exposed to isotonic (top) and then hypertonic (bottom) conditions, and GFP-ezrin mobility was estimated by fluorescence recovery after photobleaching (FRAP). Areas indicated by circles were measured for fluorescence intensity over time: circles marked with B were bleached, while circles with a C represent control areas. Data shown are representative images (a–f) and means ± SE recovery over time (g: red, hypertonic; green, control) from 10 independent experiments for each osmolarity.
ROLES OF ERM PROTEINS AFTER OSMOTIC CELL SHRINKAGE

A

Isotonic

d

Hypotonic 5'

e

f

20 μm

0.0 0.5 1.0 1.5

Relative ezrin intensity

Isotonic 5 min Hypotonic

B

ezrin

PM (tail of H-Ras)

Overlay

Isotonic

d

Hypotonic 5'

e

f

15 μm

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

Relative Ezrin-GFP intensity

Isotonic 5 min Hypotonic

C

Pre-Bleach

Post-Bleach

30 seconds

Isotonic

a

b

c

Hypotonic 5'

d

Pre-Bleach

Post-Bleach

30 seconds

d

Time (s)

% Recovery

0 0.5 1.0

0 50 100

0 100 200 300 400 500 600

1.4

300
ROLES OF ERM PROTEINS AFTER OSMOTIC CELL SHRINKAGE

Involvement of changes in cellular PtdIns(4,5)P_2 level. PtdIns(4,5)P_2 is a major regulator of ERM protein translocation (7, 23). A rapid (within 1–3 min) increase in the cellular PtdIns(4,5)P_2 level after osmotic shrinkage has, to our knowledge, been found in all cell types in which this has been studied (Refs. 21, 34, 53; see Ref. 42). Notably, an increase in the cellular PtdIns(4,5)P_2 level within seconds of osmotic shrinkage was demonstrated in the closely related Ehrlich ascites tumor cells (21, 35). To examine the role of PtdIns(4,5)P_2 in shrinkage-induced ERM protein activation, ELA cells were cotransfected with GFP-tagged ezrin and the RFP-tagged PH domain of PLC_6 (PH-PLC_6), which specifically binds PtdIns(4,5)P_2 (47). Consistent with previous reports (Refs. 34, 53; see Ref. 42), cell shrinkage appeared to increase the PtdIns(4,5)P_2 level in the plasma membrane (Fig. 5A). To dephosphorylate, and hence deplete the cells of, PtdIns(4,5)P_2, the cells were additionally transfected with a construct consisting of the phosphoinositide 5'-phosphatase domain of synaptophas, which fused to the CAAAX motif containing COOH terminus of K-ras (PD-CAAX) (31). This method was previously demonstrated to deplete the plasma membrane of PtdIns(4,5)P_2 (54). As seen (Fig. 5B), in the presence of PD-CAAX RFP-PH-PLC_6 fluorescence in the ELA cell plasma membrane was strongly reduced, consistent with a loss of PtdIns(4,5)P_2, and no increase in RFP-PH-PLC_6 fluorescence was seen on osmotic shrinkage. Under these conditions, shrinkage-induced translocation of GFP-ezrin to the cortical region was prevented (Fig. 5B). It should be noted that the presence of RFP-PH-PLC_6 in itself reduced the fraction of ezrin associated with the plasma membrane (compare Fig. 5A and Fig. 2, A and B). This presumably reflects the fact that RFP-PH-PLC_6 binds with high affinity to PtdIns(4,5)P_2, thus effectively sequestering PtdIns(4,5)P_2 and preventing its interaction with ezrin (Fig. 5A).

Together these findings demonstrate that PtdIns(4,5)P_2 is required for the shrinkage-induced translocation of ezrin and are consistent with the interpretation that a shrinkage-induced increase in PtdIns(4,5)P_2 is essential for the ezrin translocation.

Involvement of Rho-Rho kinase pathway. RhoA plays an important role in ERM protein activation by many, although not all, stimuli (56), whereas Rac1 has been assigned both activating (29) and inhibitory (36) roles. Hence, it was of interest to address the potential involvement of these pathways in shrinkage-induced modulation of ERM protein activity. In ELA cells, Rho was rapidly (within 1 min) and transiently activated by osmotic shrinkage (Fig. 6A). ERM protein phosphorylation was strongly increased by transient transfection of ELA cells with constitutively active Rho (Fig. 6B). In ELA cells pretreated with the Rho kinase inhibitor Y-27632 (10 μM, 30 min), the basal level of ERM protein phosphorylation was substantially increased compared with that in isotonic control cells and decreased rather than increased on osmotic shrinkage but remained above the level in shrunken control cells (Fig. 6B). Comparable findings were obtained in LLC-PK1 cells (n = 3; data not shown). To further evaluate the possible involvement of Rho family G proteins, ERM protein phosphorylation was monitored in NIH3T3 cells stably expressing constitutively active RhoA and Rac1, respectively. Similar to ELA cells, NIH3T3 cells also express all three ERM proteins as well as merlin, as evaluated by PCR analysis (n = 3; not shown). As seen, the pattern of shrinkage-induced ERM protein phosphorylation in wild-type NIH3T3 cells was comparable to that in ELA cells, and the relative increase in shrinkage-induced ERM protein phosphorylation in cells stably expressing constitutively active RhoA and Rac1 was similar to that in wild-type NIH3T3 cells (Fig. 6C).

Thus, although elevation of Rho activity does increase basal ERM protein phosphorylation at least in ELA cells, the Rho-
Rho kinase pathway does not appear to mediate the shrinkage-induced increase in ERM protein phosphorylation.

Consequences of Osmotic Shrinkage-Induced Ezrin Activation

Given the rapidity (<1 min) of their activation by osmotic shrinkage, we addressed the possible role of ERM proteins upstream of other major shrinkage-induced events, namely, NHE1 activation, Rho activation, reorganization of the actin cytoskeleton, and shrinkage-induced cell death. Again, we focused specifically on ezrin, for the reasons given above.

Lack of involvement of ezrin in shrinkage-induced cell death. In kidney epithelial cells, NHE1 activation by shrinkage and other apoptotic stimuli was recently proposed to elicit recruitment and phosphorylation of ezrin and activation of PI3K and the PKB survival pathway (51). The lack of dependence of shrinkage-induced ezrin activation on NHE1 (Fig. 4) contrasted with these findings, and we therefore investigated the possible involvement of this pathway in further detail. We and others have shown that prolonged osmotic cell shrinkage induces cell death (Refs. 5, 37; see Ref. 8). Consistent with this notion, osmotic shrinkage was associated with a significant reduction in the number of viable cells (MTT assay) at 24 h and 48 h after the onset of hypertonic exposure, compared with isotonic control conditions (slightly smaller in the cells transfected with either control or ezrin siRNA, presumably reflecting that the prestress of the transfection procedure exerts a slight protective effect). Compared with cells transfected with scrambled, GC-matched control siRNA, siRNA-mediated knockdown of ezrin in ELA cells resulted in an ~80% reduction in the cellular ezrin level (see Fig. 7A, top). The shrinkage-induced cell death was unaffected by ezrin knockdown as well as by preincubation with wortmannin to inhibit PI3K, arguing against a role for the ezrin-PI3K-PKB pathway (Fig. 7A). Further supporting the lack of involvement of such a pathway, PKB activity was modestly inhibited rather than activated by osmotic shrinkage of ELA cells and was largely unaffected by EIPA (Fig. 7B).

Inhibitory role of ezrin on shrinkage-induced NHE1 activation. We next assessed whether shrinkage-induced NHE1 activation might be downstream of ezrin activation. Interestingly, ezrin knockdown was associated with an ~25% increase in the rate of shrinkage-induced intracellular alkalinization in nominally HCO3−-free Ringer [previously shown to be due to NHE1 activity exclusively (39)] compared with cells transfected with control siRNA (Fig. 8A). Identical results were obtained in COS-7 cells (Fig. 8B). Together, these data indicate that ezrin counteracts or reduces the hyperosmolarity-induced activation of NHE1.

Involvement of ezrin in shrinkage-induced RhoA activation and F-actin reorganization. The results presented above demonstrated that cell shrinkage increased RhoA activity in ELA...
cells (Fig. 6A) and that expression of constitutively active RhoA increased ERM protein activity (Fig. 6B, top). However, ERM proteins may, conversely, stimulate RhoA activity by interacting with Rho-GDI (49), and therefore we next investigated this possibility. As seen, siRNA-mediated ezrin knockdown reduced shrinkage-induced RhoA activation in ELA cells by ~50% and also significantly attenuated basal RhoA activity (Fig. 9A).
In most cell types studied, osmotic shrinkage is associated with a reorganization of the actin-based cytoskeleton, which is at least in part dependent on RhoA activation (11, 42). Since ezrin associates with F-actin and is an important modulator of F-actin organization after some stimuli (29), the possible role of ezrin upstream of these changes was investigated. While cortical F-actin fluorescence in ELA cells was increased by osmotic shrinkage (Fig. 2), the shrinkage-induced increase in net cellular F-actin content was not statistically significant, and neither this increase nor the isotonic net cellular F-actin levels were significantly affected by ezrin siRNA (Fig. 9B). In ezrin siRNA-transfected cells, the shrinkage-induced formation and/or elongation of the microvillus-like F-actin protrusions appeared to be attenuated, as visualized by fluorescence imaging (Fig. 9C). Supporting this conclusion, preliminary SEM imaging of ezrin siRNA-transfected cells indicated a partial loss of microvillus-like protrusions under isotonic conditions and a marked attenuation of the shrinkage-induced increase in protrusion number and length (n = 2; data not shown). On the other hand, although net phospho-ERM protein fluorescence was reduced by ezrin knockdown, protrusions containing phospho-ERM proteins were still readily detectable in essentially all cells (Fig. 9Cd, inset; note also the distribution of phospho-ERM fluorescence to the distal part of the protrusions).

Together, these findings imply that after osmotic shrinkage of ELA cells, shrinkage-induced ezrin activation attenuates NHE1 activity, contributes to RhoA activation, and may contribute to F-actin reorganization, while no effect on shrinkage-induced cell death was detectable.

**DISCUSSION**

ERM proteins link F-actin to integral plasma membrane proteins including NHE1 and have been implicated in the regulation of Rho activity, cytoskeletal organization, and cell death/survival balance (7, 17, 23). Important mechanisms of ERM protein activation include elevated cellular PtdIns(4,5)P2 and/or RhoA activation (7, 23), events occurring rapidly after osmotic shrinkage (11, 22). We therefore hypothesized that activation of ERM proteins might be an early signal of osmotic cell perturbation, and that ezrin might in turn play important roles in the physiological consequences of cell shrinkage.

ERM protein phosphorylation on Thr567/Thr564/Thr558 was rapidly and transiently increased by hypertonic cell shrinkage in all of the investigated cell types (ELA, COS-7, NIH3T3, LLC-PK1, and AP1). The increase in ERM protein phosphorylation was almost exclusively seen in the cortical region and was particularly prominent in microvillus-like protrusions, which increased substantially in number and apparently also in length in the shrunken cells. In congruence with this, it was previously reported that osmotic swelling of PC12 cells results in a decrease in the number and length of surface microvilli.
(12), and changes in microvillus-like surface invaginations on osmotic perturbation have been reported in Ehrlich ascites tumor cells (20). However, ERM proteins have been implicated in the formation of a variety of F-actin-containing cellular protrusions (27, 45, 46, 50), and the precise identity of the shrinkage-induced protrusions remains to be determined. In contrast to the marked cortical/protrusion localization, phospho-ERM proteins did not colocalize with stress fibers, in agreement with the previously reported lack of ERM proteins from these structures (45). We therefore specifically addressed the effects of osmotic shrinkage on this ERM protein and found that cell shrinkage also elicited the translocation of ezrin to the cortical region. FRAP experiments showed that cell shrinkage rendered a fraction of the ezrin immobile and reduced the mobility of the rest of the ezrin pool. These findings are in accordance with previous FRAP experiments of ezrin mobility suggesting that the fully immobile fraction reflects the cytoskeleton-tethered, activated state of this protein, whereas the reduced mobility of...
the mobile fraction may reflect translocation to the confined space of microvillus-like structures (13). In agreement with these findings, shrinkage-induced phosphorylation of ERM proteins was shown by immunoblotting in kidney epithelial cells (51); however, the present study is to our knowledge the first to demonstrate the rapid, shrinkage-induced translocation of ERM proteins, and specifically ezrin, to the plasma membrane.

The shrinkage-induced translocation of ezrin to the plasma membrane occurred both at increased and unaltered extracellular ionic strength and was independent of the HCO$_3^-$ concentration, and hence presumably was not secondary to shrinkage-induced intracellular alkalization (note that the experiments employing AP1 cells or EIPA also suggest that an alkaline pH$_i$ is not necessary for this phenomenon). However, ezrin translocation was abolished when the plasma membrane was depleted of PtdIns(4,5)P$_2$. The cellular level of PtdIns(4,5)P$_2$ is increased within a few minutes of osmotic shrinkage in, to our knowledge, all cell types studied, including Ehrlich ascites tumor cells, the closely related parent cell line of ELA cells (Refs. 21, 34, 53; see Ref. 42). Thus, in conjunction with the fact that PtdIns(4,5)P$_2$ elevation is a well-established mechanism for ERM protein activation after a range of other stimuli (7, 23), the present findings imply that the shrinkage-induced increase in PtdIns(4,5)P$_2$ is the signal initiating ERM protein phosphorylation. RhoA was activated within 1 min of osmotic shrinkage of ELA cells, in agreement with findings in kidney tubular cells (10). Transient expression of constitutively active RhoA dramatically increased ERM protein phosphorylation, consistent with previous reports of Rho-dependent ERM protein activation (Ref. 32; see Ref. 23). Surprisingly, in both ELA and LLC-PK$_1$ cells, preincubation with the Rho kinase inhibitor Y-27632 increased ERM protein phosphorylation under basal conditions, followed by a relative decrease on shrinkage. While the mechanism whereby Y-27632 increases basal ERM phosphorylation remains to be elucidated, this phenomenon seems to be a general one, since increased ERM phosphorylation on Rho kinase inhibition by either Y-27632 or HA-1077 was also observed in the cleavage furrow of U251 glioma cells (26). Regardless of the mechanism by which inhibition of Rho kinase increases ERM protein phosphorylation, this observation does not support a role for Rho kinase in shrinkage-induced ERM protein phosphorylation, consistent with a substantial number of studies arguing against a role for Rho kinase in ERM protein phosphorylation in vivo (Ref. 32; see Ref. 23). While not further addressed here, other possible mediators of the shrinkage-induced ERM protein phosphorylation are PKC-$\alpha$ and PKC-$\beta$ (because both conventional PKCs and novel PKCs have been shown to be activated by osmotic shrinkage; see Ref. 58) and NIK (3), a member of the ste20-related kinase family, several of which are volume sensitive (see Ref. 22).

An important finding of this study is that ezrin plays a significant role in the shrinkage-induced RhoA activation. This conclusion is based on our observation that hypertonicity-evoked Rho activation was reduced by 50% on ezrin knock-
down. Both activation of RhoA and ERM protein phosphorylation are fast processes that occur within 1 min of hypertonic exposure, and their exact kinetics cannot be resolved with the present methods. Hence, we cannot exclude that RhoA also modulates ezrin activity, thus creating a feedback loop between activation of RhoA and ERM in osmotically shrunken cells; however, this remains to be determined.

Ezrin associates directly with the COOH-terminal cytoplasmic tail of NHE1 in vivo, and this interaction has been proposed to regulate cytoskeletal organization (16). Additionally, ezrin has been shown to recruit PI3K and regulate PKB (18). It was recently proposed that the NHE1-dependent recruitment and activation of ezrin counteracts cell death after osmotic shrinkage and other apoptotic stimuli by activating the PKB pathway (51). In the present study, multiple lines of evidence argue against such a scenario: 1) shrinkage-induced ERM protein phosphorylation was unaffected by EIPA in ELA cells and was similar in NHE1-deficient and NHE1-expressing AP1 cells; 2) PKB was modestly inactivated rather than activated by osmotic stress in ELA cells, in agreement with findings in other cell types (33); 3) PKB activity was modestly reduced rather than increased by inhibition of NHE1; and
neither ezrin knockdown nor PI3K inhibition significantly affected shrinkage-induced cell death. We conclude that in ELA cells, shrinkage-induced ERM protein phosphorylation occurs independently of NHE1 and cell shrinkage does not activate an NHE1-ezrin-PI3K-PKB survival pathway. The reason for the apparent differences between kidney epithelial cells and ELA cells is not clear. However, in the former, ion translocation by NHE1 was required for protection against shrinkage-induced apoptosis (52) but not for PKB activation (51), suggesting that also in these cells the role of the PKB pathway may be relatively minor.

The present study demonstrates for the first time that ezrin negatively regulates shrinkage-induced NHE1 activity in both ELA cells and COS-7 cells. Of note, ezrin has conversely been reported to stimulate the activity of the shrinkage-inhibited (Ref. 24; see Ref. 2) Na\(^+\)/H\(^+\) exchanger NHE3 (57). While this interesting reciprocity suggests a possible link with cell volume, it is not obvious why ezrin, which is activated by shrinkage, would inhibit a shrinkage-activated transporter and stimulate a shrinkage-inhibited transporter. The possible physiological relevance of this requires further investigation. Similarly, the mechanisms by which ezrin negatively regulates NHE1 function remain to be elucidated. Shrinkage-induced NHE1 activity in ELA cells was unaffected by inhibition of Rho kinase (M. Rasmussen and S. F. Pedersen, unpublished observation), in agreement with findings in several other cell types (see, e.g., Ref. 11). Hence, in osmotically shrunken cells, a role for NHE1 regulation by Rho-Rho kinase can be excluded. NHE1 activity is likely dependent on conformational changes in the cytosolic tail region, and conceivably, tethering of NHE1 to ERM proteins and via them to the cortical cytoskeleton may partially counteract such changes. ERM protein-mediated regulation of the subcellular localization of NHE1, as suggested by Denker et al. (16), may also be relevant in modulating NHE1 function. In accordance with this, ERM proteins were proposed to be involved in STAT1-mediated NHE1 inhibition, apparently by interfering with the function of NHE1 proteins in the plasma membrane (30).

ERM proteins are important regulators of cytoskeletal organization, and their activation has been shown to be essential for the formation of microvilli in various cell types (Refs. 46, 50; see Ref. 7). Substantiating this notion, the ezrin-knockout mouse displays malformed microvilli (4). In congruence with such a role, phosphorylated ERM proteins colocalized with F-actin in the shrinkage-induced microvillus-like apical protrusions, the formation of which appeared to be partially prevented by ezrin knockdown. This suggests that activation of ezrin is likely to contribute to the formation and stability of these protrusions. The inhibition of protrusion formation by ezrin knockdown was, however, only partial. While this may in part reflect incomplete siRNA-mediated knockdown, it is also in accordance with the known functional redundancy of ERM proteins, specifically the reported roles not only of ezrin but also of radixin and moesin in formation of microvillus-like protrusions in other fibroblast cell lines (46). Regardless of which ERM isoform(s) are involved, such a role of ERM proteins would be in agreement with the previously reported roles of PtdIns(4,5)P\(_2\) (53) and Rho (11) in shrinkage-induced F-actin reorganization.

In conclusion, ERM proteins are activated by osmotic shrinkage in a PtdIns(4,5)P\(_2\)-dependent, NHE1-independent manner. This in turn attenuates shrinkage-induced NHE1 activation, augments Rho activity, and may also contribute to F-actin rearrangement, while no evidence was found for the involvement of an NHE1-ezrin-PI3K-PKB pathway in counteracting shrinkage-induced death in ELA cells.

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