OSMOTIC CELL SHRINKAGE ACTIVATES EZRIN/RADIXIN/MOESIN (ERM) PROTEINS: ACTIVATION MECHANISMS AND PHYSIOLOGICAL IMPLICATIONS

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Running title: Mechanisms and consequences of shrinkage-induced ERM protein activation

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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 (ELA) cells elicited the formation of long microvillus-like actin-containing protrusions, rapid translocation of endogenous ERM proteins and GFP-tagged ezrin to the cortical region including these protrusions, and Thr^{567/564/558} (ezrin/radixin/moesin) phosphorylation of cortical ERM proteins. Shrinkage-induced ERM protein activation was unaffected by extracellular ionic strength and HCO_3^- concentration, but was prevented after PtdIns(4,5)P_2 depletion, by expression of the phosphatase domain of synaptojanin 2. Transient expression of constitutively active RhoA increased basal ERM protein phosphorylation, yet the Rho-Rho kinase pathway did not appear to be involved in the shrinkage-induced increase in ERM protein phosphorylation. Shrinkage-induced ERM protein phosphorylation was unaffected by NHE1 inhibition by EIPA, and did not require the presence of NHE1 (as evaluated in wild type NHE1-deficient AP1 cells and AP1 cells expressing human NHE1). Ezrin knockdown by siRNA increased shrinkage-induced NHE1 activity, reduced basal and shrinkage-induced Rho activity, and appeared to attenuate 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)P_2-dependent, NHE1-independent 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 death.

**Key words:** RhoA, NHE1, ezrin, osmotic stress, cytoskeleton, PtdIns(4,5)P_2
INTRODUCTION

The ezrin/radixin/moesin (ERM) protein family crosslinks plasma membrane proteins with F-actin and plays a major role in the control of cell morphology, migration, adhesion, and a variety of cellular signalling processes (reviewed in [1-3]. Notably, recent evidence has assigned important roles for ERM proteins in cancer development and metastasis [4]. 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 N-terminal FERM (four-point-one, ezrin/radixin/moesin) domain [1]. ERM proteins are maintained in an inactive cytosolic state by intramolecular association between the FERM domain (also called the N-terminal ERM association domain, or N-ERMAD) and a C-terminal region, the C-ERMAD [1,2]. In this closed conformation, the C-terminal F-actin binding site as well as the N-terminal binding sites for at least some of the ligands are masked [1]. In their activate state, ERM proteins are able to associate with F-actin through a C-terminal site, and with integral membrane proteins (either directly, or indirectly e.g via. the EBP50 family of scaffolding proteins) through the FERM domain. Two events appear to be required for ERM protein activation: interaction of phosphatidyl-inositol(4,5)bisphosphate (PtdIns(4,5)P$_2$) with the FERM domain, and phosphorylation of Thr$^{567}$ (ezrin)/Thr$^{564}$ (radixin)/Thr$^{558}$ (moesin) in the C-terminal region [1,2]. It is thought that PtdIns(4,5)P$_2$ is required for the activation and translocation of ERM proteins to the membrane, while phosphorylation is required for maintaining the active state [5].

Several ser/thr protein kinases, including protein kinase C (PKC)$\alpha$ [6], PKC$\theta$ [7], myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK, [8], G protein coupled-receptor kinase 2 (GRK2, [9], and the ste20-related kinase, Nck-interacting kinase (NIK) [10] have been proposed to mediate the threonine phosphorylation of ERM protein in vitro and/or in vivo. 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)P$_2$ [11,12], 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) [13].
Once activated, ERM proteins play important roles in control of cytoskeletal organization, and hence cell morphology, adhesion, and migration [14-16; see 17]. 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 [10,14,18-20; see 1]. ERM proteins are also implicated in the control of cell death/survival balance [21,22; see 23], and in regulation of the localization and activity of membrane transport proteins [24,25; see 3]. Cell shrinkage leads to rapid activation of RhoA in LLC-PK1 kidney epithelial cells [26] and has been shown to rapidly increase PtdIns(4,5)P2 levels in several cell types [27,28; see 29]. Taken together, this lead us to hypothesize that ERM proteins might be activated by osmotic stress and act as upstream regulators of dynamic cytoskeletal rearrangements under these conditions.

One of the plasma membrane proteins with which ezrin associates is the ubiquitous plasma membrane Na\(^{+}/H^{+}\) exchanger, NHE1 [15,16]. Direct interaction of NHE1 with ezrin has been shown to play a central role in cell migration and organization of the cortical cytoskeleton [15,16], and was recently assigned a role in NHE1-dependent protection from apoptotic cell death [22]. Osmotic cell shrinkage rapidly activates NHE1, elicits reorganization of the actin-based cytoskeleton, and, on a longer time scale, leads to cell cycle arrest and programmed cell death (see [30-32]). It has recently been suggested that ERM protein recruitment to NHE1 plays a role in the regulation of cell survival after osmotic stress and other apoptotic stimuli [22], however, the effect of osmotic shrinkage on ERM protein activity and the role of ERM proteins in mediating intracellular responses induced by osmotic cell shrinkage remains to be elucidated.

Here, we tested the hypothesis that activation of ERM proteins, and of 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 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 death.

Part of these findings have previously been published in abstract form [33].
EXPERIMENTAL PROCEDURES

Reagents and solutions

Reagents were of the highest analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA) or Mallinckrodt Baker B.V. (Deventer, NL) unless otherwise stated. 5´-(N-ethyl-N-isopropyl)amiloride (EIPA) and 2´,7´-bis-(2-carboxyethyl)-5,6-carboxyfluorescein, tetracetoxymethylester (BCECF-AM) were from Molecular Probes (Leiden, the Netherlands), and were dissolved at 2.5 mM in ddH$_2$O, and 1.2 mM in desiccated DMSO. Nigericin (Sigma) was dissolved at 5 mM in EtOH. Y27623 was from Calbiochem (Darmstadt, Germany), and was dissolved at 5 mM in ddH$_2$O, and wortmannin (Sigma-Aldrich) was dissolved at 1 mM in DMSO. PDGF-BB was also from Calbiochem, and was dissolved at 100 μg/ml in dilute HCl.

All these stock solutions were stored at -20°C. Stock solutions of paraformaldehyde (20% w/v in ddH$_2$O) were prepared fresh regularly and stored at 4°C. Monoclonal anti-ezrin antibody was from Sigma (St. Louis, MO, USA), and antibodies recognizing total ezrin/radixin/moesin (ERM) and ezrin, radixin, and moesin phosphorylated at Thr$^{567}$, Thr$^{564}$, and Thr$^{558}$, respectively (corresponding to the active state of these proteins) were from Cell Signaling (Beverly, MA), as was the antibody against protein kinase B (PKB) phosphorylated at Ser$^{473}$.

The standard Ringer’s solution for ELA cells contained (in mM) 143 NaCl, 5 KCl, 1 MgSO$_4$, 1 Na$_2$HPO$_4$, 1 CaCl$_2$, 3.3 MOPS, 3.3. TES, and 5 HEPES; pH 7.4, 310 mOsm. The standard hypertonic (600 mOsm) Ringer’s solution was prepared by doubling the concentrations of all components except MOPS, TES and HEPES compared to the standard medium, or, where indicated, by addition of mannitol to the isotonic medium. In HCO$_3^-$ media, 25 mM of NaCl was replaced by 25 mM NaHCO$_3$. 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/5% CO$_2$ in a humidified incubator. Cells were passaged every 3-4 days and only passages 6-30 were employed. All media were supplemented with 10% foetal calf serum and 1% penicillin/streptomycin solution (except for media for COS-7 cells, which did not contain penicillin/streptomycin). The cell cultures and culture media used were: Ehrlich Lettre Ascites (ELA) cells (American Type Culture Collection (ATCC), Manassas, VA, USA), maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA); Wild type NIH3T3 cells (clone 7), a kind gift from Prof. B. Willumsen, Inst. of Molecular Biology and Physiology, University of Copenhagen, and NIH3T3 cells
stably expressing constitutively active Rac1 and RhoA, prepared as previously described [34] and maintained in Dulbecco’s modified Eagle’s medium (DMEM); AP1 cells and S5 cells (AP1 cells stably expressing the human (h)NHE1, a kind gift from Prof. P.M. Cala, School of Medicine, University of California, Davis) and maintained in α-modified Eagle’s medium with 1% L-glutamine and, for transfected cells, 400 μg/ml G418-sulphate (GIBCO BRL, Invitrogen, Carlsbad, CA). 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 prior to experimentation.

* Constructs and transient transfection

The plasmid containing ezrin-GFP was a gift of Dr. Antony Bretschler, Cornell University, Ithaca, NY. The plasmid containing the PD (phosphatase domain) of synaptojanin 2 was a gift of Dr. Marc Symons [35], and plasmids containing mRFP-PH-PLC and mRFP-H-Ras were gifts of Dr. S. Grinstein [36]. The above constructs were expressed in ELA cells using, 2 μg of DNA and Lipofectamine 2000, following the manufacturer’s instructions. Experiments were performed 24-48 hrs after transfection. Myc-tagged constitutively active Rho (RhoQ63L) [37] was expressed in ELA cells grown in 10 cm Petri dishes, using Lipofectamine 2000 (Invitrogen) and 4 μg of DNA pr dish, and these cells were used for experiments 48 h after transfection.

* siRNA-mediated knockdown of ezrin

Desalted, annealed, 21-bp siRNA duplexes with 2-nt overhangs were obtained from Dharmacon RNA Technologies (Lafayette, CO, USA). 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 using 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 using Superscript II reverse transferase and random primers (Invitrogen). PCR was carried out using Taq DNA polymerase (Invitrogen), and the following protocol: 95 °C 2 min, 35 x (95 °C 30 s, 40 °C 30 s, 72 °C 2 min), 72 °C 10 min. Primer sequences used were: ezrin: fw 5’-ACAGCAGTTGGAAACCG-3’, rv 5’-GGCCTCCAGACGTTCAG-3’ (expected product
size 213 bp), radixin: fw 5’-GGCCTGAATATTATATGAACA-3’, rv 5’-AGCTCTTCCTTTTACGCTTC-3’ (expected product size 370 bp); moesin: fw 5’-TGCTCTCCCTGGAAATGAGA-3’, rv 5’-CGAGAAGCCCTGCAGCA-3’ (expected product size 263 bp); merlin: fw 5’-GAGCCAGGGATGACAGC-3’, rv 5’-TCTTGTACGCTCGGCCT-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+ DNA markers (Invitrogen), and visualized using 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 cleared by centrifugation. After protein determination (BCA protein kit, Bio-Rad, Hercules, CA, USA), 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 TBST (0.01 M Tris-HCl pH 7.4, 0.15 M NaCl and 0.1% Tween 20) containing 5% non-fat 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 using BCIP/NBT (Kirkgaard and Perry Laboratories, Gaithersburg, MD). Membranes were scanned and band intensity was estimated from arbitrary densitometric values obtained using UN-SCAN-IT software.

**Immunocytochemistry**

Cells were grown on HCl- and EtOH-washed no. 1 glass coverslips. Prior to experiments, cells were washed in isotonic Ringer’s 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 room temperature, 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 2U/ml rhodamine-conjugated phalloidin as indicated, after which they were washed extensively in TBS, and mounted with N-propyl-galleate 2% w/v in PBS/glycerine. Fluorescence was visualized using a 40 X/1.25 NA or a 100 X/1.4 NA plan...
apochromat objective and the 488 and 568 nm laser lines of a Leica DM IRB/E microscope with a Leica TSC NT confocal laser scanning unit (Leica Lasertechnik GmbH, Heidelberg, Germany). Optical slice thickness was 1 μm, and pinhole size 1 airy disc. Images shown are frame averaged and presented in RGB pseudocolor. Essentially no labeling was detectable in the absence of primary antibody (not shown). Quantification of cortex/cytoplasm ratios of endogenous ezrin was performed by defining regions of interest (ROI) in the cytoplasmic and cortical regions (the latter identified from the co-labeling for cortical F-actin labeling). The average pixel intensity per ROI was determined, and data were normalized to the cytosolic values.

**Live cell imaging of ezrin-GFP**

ELA cells were grown on 25 mm glass coverslips and transfected with Lipofectamine 2000 with the indicated constructs, 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 using Volocity™ V3.6 (Improvision) software. After taking images under the isotonic conditions at 37°C, the medium was changed to hypertonic medium, and the cells were imaged at the times indicated. Quantification of cortex/cytoplasm ratios of GFP-ezrin was performed as above, except that the cortical region was identified from co-expression of mRFP-H-Ras (Fig. 3) or mRFP-PH-PLC (Fig. 6). The average pixel intensity per ROI was determined, and data were normalized to the cytosolic values.

**Fluorescence recovery after photobleaching (FRAP)**

Experiments were performed and analyzed as described previously [38]. In brief, ELA cells were transfected with ezrin-GFP. Samples placed in Attofluor chambers were mounted on the stage of a confocal laser microscope (Zeiss LSM 510) and bathed in either isoosmolar or hyperosmolar solution. A juxtamembrane region was brought into focus and two equal areas (2 μm diameter) defined. Five minutes after being bathed in either isosomolar or hyperosmolar solution, two baseline fluorescence measurements were acquired, then one of the selected areas was irreversibly photobleached and the fluorescence of both areas measured over time. The fractional fluorescence recovery of the bleached area was determined relative to the average of the two pre-bleach measurements. The unbleached area was used to estimate possible bleaching incurred during image acquisition. FRAP data were fitted by nonlinear regression analysis using the equations by Yguerabide et al. [39].
**Rho activity measurements**

Cellular Rho-GTP levels were estimated using a commercial assay (Cytoskeleton Inc., Denver, CO, USA). Cells were grown in 10 cm Petri dishes, and in some cases transfected with ezrin siRNA 72 h prior to experiments as indicated above. After exposure to iso- or hypertonic Ringer for the time indicated, cells were quickly washed in PBS, lysed in 500 μl lysis buffer containing protease inhibitors, scraped off, and cleared by centrifugation (20,000 x g for 5 min at 4°C). 500 μl cleared lysate was transferred to 20 μl RhoA-binding domain (RBD) bead solution, 5 μl protease inhibitor cocktail was added, and lysate and beads were incubated at constant rotation for 1h at 4°C. The beads were washed once in lysis binding buffer and once in washing buffer (5,000 x g, 3 min, 4°C). RBD beads with bound Rho-GTP were spun down as above, the supernantant carefully removed, and the RBD bead pellet dissolved in 10 μl NuPage LDS sample buffer (Invitrogen) containing 29% DTT, and Rho-GTP content was analyzed by SDS-PAGE and Western blotting as described below. The remaining lysate was used for protein determination and estimation of total Rho by Western blotting. For positive controls, cells were transfected with Myc-tagged constitutively active Rho (RhoQ63L), using Lipofectamine 2000 (Invitrogen), and 4 μg DNA per 10 cm dish. 48 h after transfection, the cells were lysed and Rho-GTP isolated as described above.

**Estimation of Na⁺/H⁺ exchanger activity**

**ELA cells.** ELA cells were grown to confluence on rectangular (10 x 50 mm) HCl- and ethanol-cleaned glass coverslips. Cells were incubated for 30 min at 37°C with 1.2 μM BCECF-AM in the standard Ringer’s solution, washed twice in this solution, and a further incubated in this solution for 15 min prior to experiments. Coverslips with cells were mounted in the stirred, thermostatted cuvette of a PTI Ratiomaster spectrophotometer type C-44, at a 50° angle relative to the excitation light. Emission was detected at 525 nm after excitation at 445 nm and 495 nm, and the 445 nm/495 nm ratio was calculated after subtraction of background fluorescence (unloaded cells in the relevant experimental solution). Calibration to pHᵢ values was performed using a 7-point nigericin/high K⁺ calibration, based on [40], and modified as described earlier [41]. As 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.** Cell shrinkage induced activation of NHE1 was assessed in COS-7 cells as the hypertonicity-induced pHᵢ increase. Dual excitation ratio determinations of the fluorescence
of BCECF were used to measure pH\textsubscript{i}, as previously detailed [42]. Briefly, cells grown on 25 mm glass cover slips and transfected with siRNA were placed in Attofluor cell chambers and mounted on the stage of the microscope. Next, they were loaded with 5 \( \mu \)g/mL BCECF acetoxymethyl ester in isotonic medium at 37°C for 10 min. Extracellular dye was then washed away and Na\textsuperscript{+}/H\textsuperscript{+} exchange was initiated by introduction of the hypertonic solution. Intracellular pH (pH\textsubscript{i}) was calibrated by equilibrating the cells with K\textsuperscript{+}-rich media titrated to defined pH values and containing 10 \( \mu \)g/mL nigericin.

**Quantification of cellular F-actin levels**

Net F-actin content was estimated using a quantitative rhodamine–phalloidin binding assay, essentially as described earlier [43]. Cells were seeded in 6-well polyethylene dishes at a density of 100,000 cells per well at the time of the experiment. After stimulation, cells were fixed in 2% paraformaldehyde (15 min 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-phallodin in saponin buffer (MOPS buffer plus 0.1% saponin) for 1 h, and three washes in MOPS buffer (in mM: 5 MOPS, 5 EGTA, 20 K\textsubscript{2}HPO\textsubscript{4}, 2 MgSO\textsubscript{4}, pH 6.9). Rhodamine label was extracted in 2.5 ml gradient-grade methanol per well, by gentle agitation for 30 min, the solution was transferred to a cuvette, and rhodamine fluorescence was measured (excitation 540 nm, emission 576 nm) in a PTI Ratiomaster spectrophotometer. The assay was linear in the relevant range, and specificity for F-actin was verified by competition with a 100-fold excess of unlabelled phalloidin. Data are shown as the 576 nm emission intensity after subtraction of a methanol 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 at the day of the experiment. Cell viability was estimated from the fraction of functional mitochondria using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT), as previously described [44]. 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 osmolarity of 600 mOsm compared to 300 mOsm in isotonic RPMI medium. Data from a single experiment are the mean of values from 6 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 media but no cells).
Data analysis and statistics

Data are presented as mean ± standard error of the mean (S.E.M.) 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 showed that under isotonic conditions, ERM proteins were located throughout the cytoplasm and cortical region (Fig. 1). 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 seen also in the cytosol (Fig. 1). Fig. 2 shows confocal sections through the bottom, middle, and top of isotonic (A) and osmotically shrunken (5 min) (B) ELA cells, double labeled for F-actin and phospho-ERM proteins. As seen, osmotic shrinkage greatly increased the density of long, microvillus-like protrusions, in which the phosphorylated ERM proteins colocalized with F-actin. In contrast, there was no detectable colocalization of phospho-ERM with stress fibres (images a,d,g). Cortical F-actin labeling was also increased in osmotically shrunken cells compared to isotonic control cells, consistent with the pattern observed in multiple other cell types [29,31].

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 preparations and PCR analyses for each), in agreement with previous reports that these proteins are often co-expressed in cultured cells (see [45]. Of these proteins, we were specifically interested in ezrin, since this is the only ERM protein which has previously been shown to associate with NHE1 in vivo [15,16]. Moreover, ezrin has been found to regulate another volume-sensitive NHE isoform, NHE3 [24], and to modulate RhoA activity [13], which is also volume-sensitive [37]. Endogenous ezrin exhibited a mostly cytosolic localization pattern in ELA cells under isotonic conditions, and a partial translocation towards the cortical region upon osmotic shrinkage, in the great majority of cells studied (Fig. 3A). This pattern was confirmed by transfection of ELA cells with GPF-ezrin (Fig. 3B). For unequivocal localization of the plasma membrane for the quantification of translocation, the cells in Fig. 3B were co-transfected with the RFP-tagged tail of H-Ras [36] which associates with the plasma membrane via two palmitoyl anchors. As seen, under isotonic conditions, GFP-ezrin was evenly distributed in cytosol, and upon cell shrinkage, a partial translocation to the cortical region and microvillus-like protrusions was evident.
Fluorescence recovery after photobleaching (FRAP) has previously been used to distinguish between the cytosolic and membrane-associated forms of ezrin [46]. FRAP experiments in cells transfected with GFP-ezrin demonstrated a substantial reduction in ezrin mobility in the shrunken cells (Fig. 3C). 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 half-time 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 upon osmotic cell shrinkage.

**Mechanisms of shrinkage-induced ERM protein activation**

**Lack of dependence on HCO\textsubscript{3} concentration or extracellular ionic strength**

In the experiments shown above, osmotic cell shrinkage was induced by increasing the extracellular salt concentration in the nominal absence of HCO\textsubscript{3}. Under these conditions, the shrinkage-induced NHE1 activity results in a robust intracellular alkalinization, which is abolished or greatly attenuated in the presence of HCO\textsubscript{3} [47; see 48]. Moreover, some cellular responses to osmotic stress are dependent on whether or not extracellular ionic strength is increased (e.g. [49]). We therefore considered the effects of HCO\textsubscript{3} and ionic strength, respectively, on shrinkage-induced ERM protein phosphorylation. To allow a more direct quantitative evaluation of the impact of the various conditions tested, and to verify the immunofluorescence analyses by a different method, these experiments were performed by Western blotting. Confirming the observations from the immunofluorescence experiments, osmotic cell shrinkage was associated with increased ERM protein phosphorylation as evaluated by Western blotting (Fig. 4). As seen, the increase in phosphorylation was similar whether extracellular osmolarity was increased in the absence or presence of 25 mM HCO\textsubscript{3}, arguing against the involvement of changes in pH\textsubscript{i} in shrinkage-induced ERM protein phosphorylation. The increase in ERM protein phosphorylation was slightly greater when cells were shrunken 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 (about 80 kDa) and moesin (about 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 5 μM of the NHE1 inhibitor 5´-(N-ethyl-N-isopropyl)-amiloride (EIPA) [47]. In light of the recent report that ERM proteins are phosphorylated and recruited to NHE1 in a manner inhibited by EIPA [22], we assessed whether the shrinkage-induced ERM protein phosphorylation in ELA cells is dependent on NHE1 activity. Confirming the immunofluorescence data, Western blotting demonstrated that the relative ERM protein phosphorylation level increased during the first 30 minutes after hypertonic exposure (Fig. 5A, open circles). Inhibition of NHE1 activity by EIPA did not affect the shrinkage-induced increase in ERM protein phosphorylation (Fig. 5A, black circles), indicating that this phenomenon is independent of the transport activity of NHE1 as well as the corresponding alkalinization. As ERM proteins can directly associate with NHE1 [15], 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, this was not the case, as shrinkage-induced ERM protein phosphorylation was similar in AP1 cells, which are devoid of endogenous NHE1, and in AP1 cells transfected with the human (h)NHE1 (Fig. 5B). Thus, these findings strongly suggest that shrinkage-induced ERM protein phosphorylation occurs independently of NHE1.

*Involvement of changes in the cellular PtdIns(4,5)P\(_2\) level*

PtdIns(4,5)P\(_2\) is a major regulator of ERM protein translocation (see [1,2]), and the cellular PtdIns(4,5)P\(_2\) level is rapidly (within 1-3 min) increased by osmotic shrinkage in several cell types, including Ehrlich ascites tumor cells, the parent cell line of ELA cells [27,28; see 29]. To examine the role of PtdIns(4,5)P\(_2\) in shrinkage-induced ERM protein activation, ELA cells were co-transfected with GFP-tagged ezrin and the RFP-tagged PH-domain of PLC\(\delta\) (PH-PLC\(\delta\)), which specifically binds PtdIns(4,5)P\(_2\) [50]. Consistent with previous reports [27,28;
see 29], cell shrinkage appeared to increase the PtdIns(4,5)P$_2$ level in the plasma membrane (Fig. 6A). In order to dephosphorylate, and hence deplete the cells of, PtdIns(4,5)P$_2$, a construct consisting of the phosphoinositol 5'-phosphatase domain of synaptotagmin 2 fused to the CAAX-motif containing carboxy terminus of K-ras (PD-CAAX) [35] was co-expressed with GFP-erzrin and RFP-PH-PLCδ. In the presence of PD-CAAX, RFP-PH-PLCδ fluorescence in the plasma membrane was strongly reduced, consistent with a loss of PtdIns(4,5)P$_2$, and no increase in RFP-PH-PLCδ fluorescence was seen upon osmotic shrinkage (Fig. 6B). Under these conditions, shrinkage-induced translocation of GFP-erzrin to the cortical region was prevented (Fig. 6B). It should be noted that the presence of RFP-PH-PLCδ in itself reduced the fraction of erzrin associated with the plasma membrane (compare Fig. 6A with Fig. 3A-B). This presumably reflects the fact that RFP-PH-PLCδ binds with high affinity to PtdIns(4,5)P$_2$, thus effectively sequestering PtdIns(4,5)P$_2$ and preventing its interaction with erzrin (Fig. 6A).

Taken together, these findings demonstrate that PtdIns(4,5)P$_2$ is required for the shrinkage-induced translocation of erzrin, and are consistent with the interpretation that a shrinkage-induced increase in PtdIns(4,5)P$_2$ is essential for the erzrin translocation.

**Involvement of the Rho-Rho kinase pathway**

As noted above, RhoA plays an important role in ERM protein activation by many (although not all, see [5]), stimuli, whereas Rac1 has been assigned both activating [51] and inhibitory [52] roles in ERM protein regulation. In ELA cells, Rho was rapidly (within 1 min) and transiently activated by osmotic shrinkage (Fig. 7A). ERM protein phosphorylation was strongly increased by transient transfection of ELA cells with constitutively active Rho (Fig 7B, top). In ELA cells pretreated with the Rho kinase inhibitor Y27632 (10 μM, 30 min), the basal level of ERM protein phosphorylation was substantially increased compared to that in isotonic control cells, and decreased rather than increased upon osmotic shrinkage, but remained above the level in shrunken control cells (Fig. 7B). 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. 7C).

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 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 cellular 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 were recently proposed to elicit NHE1-dependent recruitment and phosphorylation of ezrin and activation of PI3K and the PKB survival pathway [22]. The lack of dependence of shrinkage-induced ezrin activation on NHE1 (Fig. 5) 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 [44,53; see 54]. Consistent with this notion, osmotic shrinkage was associated with an about 15% reduction in the number of viable cells (as measured by MTT assay) at 24 h, and 40% at 48 h after the onset of hypertonic exposure, compared to isotonic control conditions. This shrinkage-induced cell death was unaffected by ezrin knockdown as well as by pre-incubation with wortmannin to inhibit PI3K, arguing against a role for the ezrin-PI3K-PKB pathway (Fig. 8A). 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 unaffected by EIPA (Fig. 8B).

**Inhibitory role of ezrin on shrinkage-induced NHE1 activation**

We next assessed whether shrinkage-induced NHE1 activation might be downstream of ezrin activation. The siRNA-mediated knockdown of ezrin in ELA cells resulted in an about 80% reduction in the cellular ezrin level (Fig. 9A, top). Interestingly, ezrin knockdown was associated with an about 25% increase in the rate of shrinkage-induced intracellular alkalinization in nominally HCO_3^-free Ringer (which we have previously shown to be due to
NHE1 activity exclusively, see reference [47], compared to cells transfected with GC-matched, scrambled siRNA (Fig. 9A). Identical results were obtained in COS-7 cells (Fig 9B). Taken together, these data indicate that ezrin negatively regulates NHE1 activity in osmotically shrunken cells.

**Involvement of ezrin in shrinkage-induced RhoA activation and F-actin reorganization**

Consistent with previous reports, we found that expression of constitutively active Rho increased ERM protein activity (Fig. 7B, top). However, ERM proteins may, conversely, stimulate RhoA activity by interacting with Rho-GDI [13]. Given the substantial shrinkage-induced increase in Rho activity in ELA cells (Fig. 7A), we next investigated this possibility. As seen, siRNA-mediated ezrin knockdown reduced shrinkage-induced RhoA activation in ELA cells by about 50%, and also significantly attenuated basal RhoA activity (Fig. 10A).

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 (see [29,31]). Since ezrin associates with F-actin and is an important modulator of F-actin organization after some stimuli [51], 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. 10B). In ezrin siRNA-transfected cells, the shrinkage-induced formation and/or elongation of the microvillus-like F-actin protrusions appeared to be attenuated (Fig. 10C). 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 (10C, panel d insert).

Taken together, these findings imply that after osmotic shrinkage of ELA cells, shrinkage-induced ezrin activation attenuates NHE1 activity, contributes to shrinkage-induced RhoA activation, and possibly plays a role in shrinkage-induced 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 [1-3]. ERM proteins are activated by elevated cellular PtdIns(4,5)P$_2$ and/or RhoA activation [1,2], events occurring rapidly after osmotic shrinkage [30,31]. 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 Thr$^{567}$/Thr$^{564}$/Thr$^{558}$ was rapidly and transiently increased by hypertonic cell shrinkage in ELA cells, COS-7 cells, NIH3T3 cells, LLC-PK1 cells, and AP1 cells. 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 [55], and similar changes in microvillus-like surface invaginations have been reported in Ehrlich ascites tumor cells [56]. However, ERM proteins have been implicated in the formation of a variety of F-actin containing cellular protrusions [14,18-20], 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 fibres, in agreement with the previously reported lack of ERM proteins from these structures [19].

Of the three ERM proteins, only ezrin has been shown to associate directly with NHE1 in vivo [15,16]. Moreover, ezrin regulates another NHE isoform, NHE3 [24], and may also regulate RhoA [13]. 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 [46]. In agreement with these findings, shrinkage-induced phosphorylation of ERM proteins was shown by immunoblotting in kidney epithelial cells [22], 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 alkalinization. However ezrin translocation was abolished when the plasma membrane was depleted of PtdIns(4,5)P$_2$. As the cellular level of PtdIns(4,5)P$_2$ is increased within a few minutes of osmotic shrinkage in several cell types including the parent cell line of ELA cells, EAT cells [27,28; see 29], this strongly suggests that the shrinkage-induced increase in PtdIns(4,5)P$_2$ is the signal initiating shrinkage-induced ERM protein phosphorylation. Rho was activated within 1 min of osmotic shrinkage of ELA cells, in agreement with findings in kidney tubular cells [26]. Transient expression of constitutively active RhoA dramatically increased ERM protein phosphorylation, consistent with previous reports of Rho-dependent ERM protein activation [12; see 2]. Surprisingly, in both ELA and LLC-PK1 cells, preincubation with the Rho kinase inhibitor Y-27632 increased ERM protein phosphorylation under basal conditions, followed by a relative decrease upon 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 upon Rho kinase inhibition by either Y-27632 or HA1077 was also observed in the cleavage furrow of U251 glioma cells [57]. 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 [12; see 2]. While not further addressed here, other possible mediators of the shrinkage-induced ERM protein phosphorylation are PKC$\alpha$ and PKC$\theta$ (as both cPKCs and nPKCs have been shown to be activated by osmotic shrinkage, [58], and NIK [10], a member of the ste20-related kinase family, several of which are volume-sensitive (see [30]).

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 the hypertonicity-evoked Rho activation was reduced by 50% upon ezrin knockdown. 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 current 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 C-terminal cytoplasmic tail of NHE1 in vivo, and this interaction has been proposed to regulate cytoskeletal organization [15]. Additionally, ezrin has been shown to recruit PI3K and regulate PKB [21]. It was recently proposed that the NHE1-activity dependent recruitment and activation of ezrin counteracts cell death after osmotic shrinkage and other apoptotic stimuli by activating the PKB pathway [22]. In the present study, multiple lines of evidence argue against such a scenario: (i) shrinkage-induced ERM protein phosphorylation was unaffected by EIPA in ELA cells, and was similar in NHE1-deficient and NHE1-expressing AP1 cells (ii) PKB was modestly inactivated by osmotic stress in ELA cells, in agreement with findings in other cell types [59]; (iii), PKB activity was modestly reduced rather than activated by inhibition of NHE1, and (iv) 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 pro-survival pathway. The reason for the observed differences between kidney epithelial cells and ELA cells is not clear. Notably, in kidney epithelial cells, ion translocation by NHE1 was required for NHE1-mediated protection against shrinkage-induced apoptosis [60], yet not for NHE1-dependent PKB activation [22], 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 [42; see 32] Na\(^{+}/H^{+}\) exchanger, NHE3 [24]. 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, 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 Rho kinase (M. Rasmussen and S.F. Pedersen, unpublished), in agreement with findings in several other cell types (see e.g. [31]). Hence, a role for NHE1 regulation by Rho-Rho kinase [61] can be excluded. NHE1 activation 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. [15], 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 [62].

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 [14,18; see 1]. Substantiating this notion, the ezrin knockout mouse displays malformed microvilli [63,64]. 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 [18]. 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$_2$ [28] and Rho [31] 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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References


Figure legends

Figure 1. Localization and phosphorylation of ERM proteins in ELA cells under iso- and hypertonic conditions

ELA cells grown on 22 mm glass coverslips were exposed to isotonic (300 mOsm, 10 min) or hypertonic Ringer’s solution (600 mOsm, 1 min), paraformaldehyde-fixed, permeabilized, blocked, and labeled with primary antibodies against either total (a-b) or Thr$^{567}$/Thr$^{564}$/Thr$^{558}$-phosphorylated (c-d) ERM proteins, and the relevant FITC-conjugated secondary antibodies. Fluorescence was analyzed by confocal laser scanning microscopy using a 40X/1.25 NA objective, and the images are 1 μm optical sections taken at a distance from the coverslip of approximately one-third the total cell height. Images shown are representative of 3-4 (total ERM) or 5-7 (p-ERM) independent experiments for each condition. Essentially no labeling was detectable in the absence of primary antibody (not shown).

Figure 2. Colocalization of phosphorylated ERM proteins in ELA cells with F-actin in cortex and microvillus-like protrusions, but not in stress fibres

Cells were exposed to isotonic (A, 300 mOsm, 10 min) or hypertonic (B, 600 mOsm, 5 min) Ringer’s solution, and were subsequently fixed, and labeled as described in the legend to Fig. 1, except that rhodamine-conjugated phallodin (2 U/ml) was included to label F-actin. Fluorescence was analyzed by confocal laser scanning microscopy using a 40X/1.25 NA objective, and the images are 1 μm optical sections taken at the bottom (left panel, images a, d, and g), middle (middle panel, images b, e, and h), and top (right panel, images c, f, and i) part of the cell. Images shown are representative of 4-5 independent experiments for each condition. Essentially no labeling was detectable in the absence of primary antibody (not shown).

Figure 3. Localization and shrinkage-induced translocation of ezrin in ELA cells

A. Cells were treated and imaged as described in the legend to Fig. 1, except that the antibody used was a monoclonal ezrin antibody. The images shown illustrate cells under isotonic conditions (upper panel) and after 5 min of hypertonic exposure (lower panel); a, d: ezrin, b, e: F-actin (rhodamine phalloidin), c, f: merge, g: quantification of cytoplasmic and cortical ezrin labeling. Images shown are representative of 3 independent experiments for each condition. Quantification was performed by defining regions of interest (ROI) in the
cytoplasmic and cortical regions (the latter identified from the co-labeling for cortical F-actin labeling). The average pixel intensity per ROI was determined, and data were normalized to the cytosolic values and given as mean with SEM error bars.

B. Cells were co-transfected with GFP-tagged ezrin and mRFP-H-Ras as described in Materials and Methods, exposed to hypertonic conditions for 5 min, and monitored by spinning-disk confocal microscopy. a, d: ezrin, b, e: mRFP-H-Ras, c, f: merge, g: quantification of cytoplasmic and cortical GFP-ezrin. The experiment shown is representative of 12 independent experiments. Quantification of cytoplasmic and cortical GFP-ezrin was performed as above, except that the plasma membrane was identified from the co-labeling for mRFP-H-Ras).

C. Cells were transfected with GFP-ezrin and exposed to iso- (upper panel) then hypertonic (lower panel) conditions, and GFP-ezrin mobility was estimated by fluorescence recovery after photobleaching (FRAP). The areas indicated by circles in the figure were measured for fluorescence intensity over time, the circles with a B next to them were bleached, while the circles with a C represent control areas. Data shown are representative images and the mean ± SEM of the percent recovery over time, from 10 independent experiments for each osmolarity.

**Figure 4. Shrinkage-induced ERM protein phosphorylation in ELA cells is independent of extracellular ionic strength or bicarbonate concentration**

ELA cells were exposed to isotonic (300 mOsm) conditions, or to 600 mOsm Ringer’s solution prepared by doubling the salt concentration relative to isotonic conditions in the nominal absence of HCO₃⁻ (hypertonic, salt) or by addition of mannitol (hypertonic, mannitol). Where indicated, 25 mM NaCl was replaced by 25 mM NaHCO₃ (iso- or hypertonic, HCO₃⁻). After 10 min of isotonic or 15 min of hypertonic exposure, crude membrane lysates were prepared, equal amounts of protein per well were separated by SDS-page, and total and phosphorylated ERM proteins were detected by Western blotting and quantified by densitometry scanning. Data shown are p-ERM and ERM protein labelled membranes (top) representative of 7 independent experiments, and these data summarized as mean ± S.E.M. of the p-ERM level relative to isotonic control, normalized to total ERM protein.

*) indicates that the value is significantly different from the isotonic control value.

#) indicates that the value is significantly different from the hypertonic value when cells were
shrunken in high salt.

Figure 5. Lack of involvement of NHE1 in shrinkage-induced ERM protein activation.

A. ELA cells were exposed to iso- or hypertonic conditions for the times indicated, and in the absence or presence of the NHE1 inhibitor EIPA (5 µM). Crude membrane lysates were prepared, equal amounts of protein per well were separated by SDS-page, and phospho-ERM protein detected by Western blotting and quantified by densitometry scanning. Data are from 7 independent experiments for each condition. A representative p-ERM labelled membrane is shown, and data are summarized as mean ± S.E.M. of the p-ERM level relative to isotonic control.

B. Wildtype AP1 cells and AP1 cells expressing human NHE1 were exposed to iso- or hypertonic conditions for the times indicated. Crude membrane lysates were prepared, equal amounts of protein per well were separated by SDS-page, and phospho-ERM proteins were detected by Western blotting and quantified by densitometry scanning. Data are from 6-7 independent experiments for each condition. Representative p-ERM labelled membranes are shown for each cell type, and data are summarized as mean ± S.E.M. of the p-ERM level relative to isotonic control.

*) indicates that the value is significantly different from the isotonic control value.

Figure 6. Dependence of shrinkage-induced ezrin translocation in ELA cells on plasma membrane PtdIns(4,5)P2

ELA cells were transfected with GFP-ezrin, and with either (A) the RFP-tagged PH domain of phospholipase CΔ (PH-PLCΔ) to visualize PtdIns(4,5)P2 and (B) PH-PLCΔ and the 5-phosphatase domain of synaptojanin 2 fused to the CAAX-motif containing carboxy terminus of K-ras (PD-CAAX) , which will dephosphorylate, and therefore deplete the plasma membrane of, PtdIns(4,5)P2. The cells were exposed to iso- or hypertonic conditions as indicated and monitored by spinning disk confocal microscopy. a, b: GFP-ezrin, c, d: mRFP-PH-PLCΔ, e, f: merge, g: quantification of cytoplasmic and cortical GFP-ezrin. Quantification of cytoplasmic and cortical GFP-ezrin was performed as in Figure 3, except that the plasma membrane was identified from the co-labeling for mRFP- PH-PLCΔ in A, and by using the 10 pixels at the edge of the cytosol for B. Data shown are representative of at least 5 independent experiments for each condition.
**Figure 7. Possible roles of Rho proteins and Rho kinase in regulation of ERM protein phosphorylation**

A. Rho activity in ELA cells was monitored under iso- and hypertonic conditions using a Rhotekin-based Rho-GTP pull-down assay as described in Materials and Methods. Shown are (top) representative blots of active Rho and total Rho under the conditions shown and with thrombin (1 U/ml) as a positive control for Rho activation, and Rho activity summarized as mean ± SEM of 3-13 independent experiments.

B. ELA cells were exposed to iso- or hypertonic conditions for the times indicated, with or without prior incubation (10 µM, 30 min) with the Rho kinase inhibitor Y27632. Crude membrane lysates were prepared, equal amounts of protein per well were separated by SDS-page, and phospho- and total ERM protein levels detected by Western blotting and quantified by densitometry scanning. Representative p-ERM and ERM labelled membranes are shown for iso- and hypertonic conditions in the absence or presence of Y27632, and for cells transiently expressing constitutively active (CA) RhoA (RhoQ63L). Data are summarized as mean ± S.E.M. of the p-ERM level relative to isotonic control. Data are from 6-8 (ctrl), 6 (Y27632) and 3 (CA Rho) independent experiments for each condition.

C. Wildtype NIH3T3 cells or clones expressing constitutively active Rac1 (Rac1V12) or RhoA (RhoAV14) were exposed exposed to iso- or hypertonic conditions for the times indicated, and immunoblotted as in B. Representative p-ERM and ERM labelled membranes for wild type cells are shown. Data are summarized as mean ± S.E.M. of the p-ERM level relative to isotonic control. Data are from 4-5 independent experiments for each condition.

*) indicates that the value is significantly different from the isotonic control value.

**Figure 8. Osmotic cell shrinkage does not activate a protective NHE1-ezrin-PI3K-PKB pathway in ELA cells**

A. Cells plated in 96 well plates were transfected with ezrin siRNA or control siRNA, and after 72 h, were either maintained isotonic or exposed to hypertonic conditions by addition of sterile 3 M NaCl to the growth medium to a final osmolarity of 600 mOsm. Where indicated, wortmannin (200 nM, 1 h preincubation) was present to inhibit PI3K. After 24 or 48 h, cell viability was estimated by MTT assay as described in Materials and Methods. Data are shown as mean ± SEM of the loss of cell viability relative to the corresponding isotonic condition, and are representative of 4-6 independent experiments for each condition.
B. Cells were exposed to iso- or hypertonic conditions in the presence or absence of EIPA (5 µM) for the times indicated, lysed, and equal amounts of protein per lane separated by SDS-page, and subjected to immunoblotting against protein kinase B (PKB) phosphorylated on Ser\(^{473}\). As a positive control, cells were exposed to platelet-derived growth factor (PDGF-BB, 50 ng/ml), separated and immunoblotted using the same procedures. Data are shown as a representative PKB-Ser\(^{473}\) membrane (top), and mean ± SEM of 7 independent experiments for each condition (except the PDGF control, which was repeated twice).

*) indicates that the value is significantly different from the isotonic control value.

Figure 9. Effect of ezrin knockdown on shrinkage-induced NHE1 activation in ELA and Cos-7 cells

A. ELA cells were plated on rectangular glass coverslips, and transfected with siRNA directed against mouse ezrin or with scrambled, GC-matched control siRNA as indicated. Cells were used for experiments 72 h after transfection, at which time ezrin levels were reduced by about 80% in cells transfected with ezrin siRNA, while control siRNA had no effect (top panel, immunoblotting using monoclonal ezrin antibody). For estimation of NHE1 activity, cells were loaded with BCECF and pH\(_i\) monitored over time by fluorescence spectroscopy under isotonic conditions and after hypertonic exposure (600 mOsm, salt) as indicated to activate NHE1. The initial rate of increase in intracellular H\(^+\) concentration (d[H\(^+\)]/dt) was calculated from the initial, linear part of the curve (0-90 s) after hypertonic exposure. Data shown are a pH\(_i\) trace from a single representative experiment, and d[H\(^+\)]/dt data summarized as mean with S.E.M. error bars of data from 5 independent experiments for each condition.

*) indicates that the value is significantly different from the isotonic control value.

B. Cos-7 cells were plated on 25 mm glass cover slips, transfected with ezrin or control siRNA as above, loaded with BCECF, and pH\(_i\) monitored by fluorescence microscopy and digital image analysis under isotonic conditions and after hypertonic exposure (addition of 200 mM sucrose) as indicated. After the pH\(_i\) measurements, cells were lysed, separated by SDS-page, and immunoblotted using the monoclonal ezrin antibody, shown are 3 experiments (S#1-3) from each condition. The traces shown are mean with S.E.M. error bars of data, representative of approximately 15 cells/experiment from at least 6 independent experiments for each condition.
Figure 10. Effect of ezrin knockdown on shrinkage-induced changes in Rho activity, and F-actin content and organization in ELA cells

A. 72 h after transfection with ezrin siRNA or scrambled siRNA as indicated, cells grown in 10 cm Petri dishes were subjected to isotonic (5 min) or hypertonic (1 min) Ringer’s solution, lysed, and Rho activity measured using a Rhotekin-based Rho-GTP pull-down assay as described in Materials and Methods. Shown are (left) representative blots of active Rho and total Rho under the conditions shown, and (right) Rho activity summarized as mean ± SEM of 3-13 independent experiments.

*) indicates that the value is significantly different from the isotonic control value.

#) indicates that the value is significantly different from the corresponding value in cells treated with scrambled siRNA.

B. 72 h after transfection with ezrin siRNA or scrambled siRNA, cells grown in 6-well dishes were subjected to iso- or hypertonic (5 min) Ringer’s solution, paraformaldehyde-fixed, permeabilized, and net cellular F-actin content measured using a quantitative rhodamine-phalloidin extraction assay, as described in Materials and Methods. Data shown are net F-actin content relative to the isotonic control (left), or relative to the isotonic value for the same condition (right), and are mean ± S.E.M. of 6 independent experiments for each condition.

C. 72 h after transfection with ezrin siRNA or scrambled siRNA, cells grown on 22 mm round coverslips were subjected to iso- or hypertonic (5 min) Ringer’s solution, paraformaldehyde-fixed, permeabilized, and labeled for F-actin (rhodamine-phalloidin) and phospho-ERM proteins as described in the legend to Fig. 2. Cells were visualized by confocal microscopy (100x/1.4 NA lens). Data shown are representative of 4 (p-ERM) independent experiments for each condition. Essentially no labeling was detectable in the absence of primary antibody (not shown).
Fig. 1

Total ERM

Isotonic

Hypertonic 1 min

Phospho-ERM

a

b

c

d
Fig. 3
Fig. 4

p-ERM rel. to isotonic ctrl., t=15 (norm. to total ERM)

- Iso ctrl
- Hyper, salt
- Hyper, mann
- Iso, HCO₃⁻
- Hyper, HCO₃⁻
Fig. 5

A

Hypertonic (min)

- + - + - + - + - +

p-ERM

Time (min)

0 5 15 30 45 60

Ctrl.

EIPA

B

Hypertonic (min)

iso 5 1 5 15 30 45

Wildtype

+ human NHE1

Time (min)

5 15 30 45

Wildtype

+ hNHE1

p-ERM protein level relative to isotonic ctrl.

Time (min)
Fig. 6

A. Control- with pH domain
   Isotonic Hypertonic 5'
   ezrin  
   PIP2 (pH domain of PLCβ)
   Overlay

B. + 4 Phosphatase
   Isotonic Hypertonic 5'

\[ g \]

<table>
<thead>
<tr>
<th></th>
<th>Isotonic</th>
<th>5 min Hypertonic</th>
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<tbody>
<tr>
<td>Relative Ezrin-GFP Intensity</td>
<td></td>
<td></td>
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<td>Ezrin-GFP Intensity</td>
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<td>1</td>
</tr>
<tr>
<td>Ezrin-GFP Intensity</td>
<td>0.8</td>
<td>1</td>
</tr>
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</table>

* p = 0.0026

\[ g \]

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</table>

* p = 0.083
Fig. 7
**Fig. 8**

A. Graphs showing cell viability (percent of isotonic value) for Ctrl siRNA and Ezrin siRNA at 24 h and 48 h. Ctrl. siRNA shows higher cell viability compared to Ezrin siRNA.

B. Western blot analysis of p-PKB and PKB under isotonic (iso), PDGF-BB, and hypertonic (hyper) conditions. Hypertonic treatments show an increase in p-PKB phosphorylation.

Graphs depicting relative PKB phosphorylation (norm. to total protein) over time (min) for Ctrl. and EIPA treatments. EIPA significantly reduces PKB phosphorylation compared to Ctrl. at various time points.
A

![Graph showing intracellular pH (pHi) over time with Ctrl siRNA and Ezrin siRNA](image)

- **Initial d[H+]i/dt**:
  - Ctrl siRNA: 0.0
  - Ezrin siRNA: 0.2

B

![Graph showing intracellular pH (pHi) over time with Ezrin siRNA](image)

- **Intracellular pH (pHi)** changes over time with hypertonic sucrose addition.
- **Time (s)**:
  - 0
  - 200
  - 400
  - 600

**Fig. 9**
Fig. 10

A

Isotonic Hypertonic

Active Rho

Total Rho

Ctrl. ctrlsiRNA ezrin siRNA Ctrl. ctrlsiRNA ezrin siRNA thrombin

Ctrl. Ctrl. siRNA Ezrin siRNA

Ctrl. Ctrl. siRNA Ezrin siRNA

B

Rho activity relative to isotonic ctrl.

(normalized to total Rho)

0.0 0.5 1.0 1.5 2.0 2.5 3.0

Ctrl. Ctrl. siRNA Ezrin siRNA

Ctrl. Ctrl. siRNA Ezrin siRNA

C

Isotonic Hypertonic 5’

Ctrl. siRNA Ezrin siRNA

C

Ezrin siRNA

a b c d

Fig. 10