Osmotic shrinkage elicits FAK- and Src phosphorylation and Src-dependent NKCC1 activation in NIH3T3 cells

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Submitted 6 March 2014; accepted in final form 7 October 2014

Rasmussen LJ, Müller HS, Jørgensen B, Pedersen SF, Hoffmann EK. Osmotic shrinkage elicits FAK- and Src phosphorylation and Src-dependent NKCC1 activation in NIH3T3 cells. Am J Physiol Cell Physiol 308: C101–C110, 2015. First published November 5, 2014; doi:10.1152/ajpcell.00070.2014.——The mechanisms linking cell volume sensing to volume regulation in mammalian cells remain incompletely understood. Here, we test the hypothesis that activation of nonreceptor tyrosine kinases Src, focal adhesion kinase (FAK), and Janus kinase-2 (Jak2) occurs after osmotic shrinkage of NIH3T3 fibroblasts and contributes to volume regulation by activation of NKCC1. FAK phosphorylation at Tyr397, Tyr576/577, and Tyr861 was increased rapidly after exposure to hypertonic (575 mOsm) saline, peaking after 10 (Tyr397, Tyr576/577) and 10–30 min (Tyr861). Shrinkage-induced Src family kinase autophosphorylation (pTyr416-Src) was induced after 2–10 min, and immunoprecipitation (Tyr861). Shrinkage-induced Src family kinase autophosphorylation (pTyr416-Src) was induced after 2–10 min, and immunoprecipitation indicated that this reflected phosphorylation of Src itself, rather than Fyn and Yes. Phosphorylated Src and FAK partly colocalized with vinculin, a focal adhesion marker, after hypertonic shrinkage. The Src inhibitor pyrazolopyrimidine-2 (PP2, 10 μM) essentially abolished shrinkage-induced FAK phosphorylation at Tyr576/577 and Tyr861, yet not at Tyr397, and inhibited shrinkage-induced NKCC1 activity by ~50%. The FAK inhibitor PF-573,228 augmented shrinkage-induced Src phosphorylation, and inhibited shrinkage-induced NKCC1 activity by ~15%. The apparent role of Src in NKCC1 activation did not reflect phosphorylation of myosin light chain kinase (MLC), which was unaffected by shrinkage and by PP2, but may involve Jak2, a known target of Src, which was rapidly activated by osmotic shrinkage and inhibited by PP2. Collectively, our findings suggest a major role for Src and possibly the Jak2 axis in shrinkage-activation of NKCC1 in NIH3T3 cells, whereas no evidence was found for major roles for FAK and MLC in this process.

FAK phosphorylation; Src phosphorylation; vinculin; focal adhesions; Na⁺-K⁺-2Cl⁻ cotransporter; SLC12A1; Jak2

The mechanisms linking cell volume sensing to volume regulation in mammalian cells are still incompletely understood. Integrins, which link the extracellular matrix to the cytoskeleton via focal adhesions (FAs), are essential cellular mechanosensory and mechanotransduction elements and have been assigned a role in cell volume sensing (38). The nonreceptor tyrosine kinases focal adhesion kinase (FAK) and Src are among the proteins first recruited to FAs upon integrin activation (44) and have likewise been implicated in volume sensing (15). FAK is activated by phosphorylation at multiple tyrosine residues, including Tyr397, Tyr576/577, Tyr861, and Tyr925. Tyr397 (7, 12, 35) is autophosphorylated in response to growth factor receptor stimulation and integrin clustering (7, 12), creating binding sites for Src family kinase, phosphatidylinositol-3 kinase (PI3K), growth factor receptor-bound protein 7 (Grb7), and phospholipase C γ (PLCγ), and connecting FAK to a wide variety of signal transduction cascades involved in cell migration, proliferation, and programmed cell death (PCD). Tyr576/577 is located in the kinase-activation loop, and Tyr861 is located COOH terminally (7) and recruits scaffolding proteins involved in regulation of the F-actin cytoskeleton and cell motility (26). Because of its central roles in cell migration, cell cycle progression, and PCD, FAK is an interesting anticancer target (32). Cell shrinkage has been found to stimulate phosphorylation of specific tyrosine residues on FAK in several cell types (8, 26, 27, 46). In Swiss 3T3 fibroblasts, FAK was phosphorylated on Tyr397 and Tyr576/577 after 5 min of hypertonic stress (450 mM sucrose) (27). Phosphorylation of Tyr397 occurred via a Cdc42-dependent pathway and was proposed to involve FAK aggregation and dimerization, while phosphorylation of Tyr576/577 was Src dependent (27). In these cells, functional FAK and Src were required for resistance to prolonged hypertonic stress (27). In another study, hypertonic stimulation increased the phosphorylation of FAK at Tyr861 in three types of epithelial cells, while phosphorylation of Tyr397 and Tyr576/577 was not significantly induced (26).

Similar to FAK, Src family kinases are activated downstream of activation of integrins and growth factor receptors and regulate multiple cellular processes, including proliferation and migration. Src family kinases have been assigned central roles in volume sensing, and various members of the family have been found to be activated in response to cell shrinkage (20, 23, 36) and swelling (4, 24, 45). FAK and Src physically interact and reciprocally regulate each other in a complex manner (43). Another widely expressed nonreceptor tyrosine kinase, Jak2, has also been reported to be activated by hypertonicity (10), although the mechanism involved was not elucidated.

Although the osmolyte transporters mediating the regulatory volume increase (RVI) process after osmotic cell shrinkage are much better understood than the volume sensing mechanisms, major gaps remain in the knowledge of the signaling pathways through which the transporters are activated by cell shrinkage (28). The Na⁺-K⁺-2Cl⁻ cotransporter NKCC1 plays a central role in RVI in numerous cell types (15). A role for FAK in regulation of NKCC1 was proposed in the mitochondria-rich cells of the operculum epithelium of Killifish, based on the finding that Tyr407 phosphorylation of FAK was osmosensitve and that FAK colocalized and coimmunoprecipitated with integrin β1 and NKCC1 in chloride cells (29, 30). Another kinase that has been implicated in NKCC1 activation by shrinkage is myosin light-chain kinase (MLCK) (22, 33), based

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on the inhibition of shrinkage-induced NKCC1 activity by the MLCK inhibitor ML-7, and on the shrinkage-induced phosphorylation of the only known MLCK substrate, myosin light chain (MLC), in several cell types (5, 6, 21, 33). Interestingly, NKCC1 has been shown to be activated by prolactin in a manner involving Jak2 (39); however, the possible role of Jak2 in shrinkage-activation of NKCC1 has never been explored.

In the present study, we test the hypothesis that activation of Src family kinases, FAK, and Jak2 occurs after osmotic shrinkage of NIH3T3 fibroblasts and contributes to RV1 by activation of NKCC1. We find that hyperosmotic stress induces FAK phosphorylation at Tyr397, Tyr576/577, and Tyr861, as well as c-Src phosphorylation at Tyr416, and that both activated kinases localize to focal adhesions. Also Jak2 is rapidly phosphorylated after osmotic shrinkage, in a manner sensitive to inhibition by the Src inhibitor PP2. Furthermore, PP2 strongly attenuates shrinkage-activation of NKCC1, without affecting MLC phosphorylation. In contrast, inhibition of FAK increases shrinkage-induced Src activation and only modestly attenuates NKCC1 activation. Thus our findings suggest the likely involvement of a Src-Jak2 axis in shrinkage activation of NKCC1 in NIH3T3 cells, whereas no evidence was found for major roles for FAK and MLC in this process.

MATERIALS AND METHODS

Solutions and reagents. The standard isotonic saline solution (300 mOsm) contained (in mM) 143 NaCl, 5 KCl, 1 MgSO4, 1 Na2HPO4, 1 CaCl2, 3.3 MOPS, 3.3 TES, and 5 HEPES, pH 7.4. The standard hypertonic saline (755 mOsm) was prepared by doubling the concentrations of all components of the isotonic saline solution except buffers and CaCl2. The standard hypotonic saline solution (175 mOsm) was prepared with all concentrations except buffers and CaCl2 at 50% of those in the isotonic saline. 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) was from Invitrogen and dissolved at 5 mM in ddH2O. Bumetanide was from LEO Pharma (Ballerup, Denmark) and dissolved at 10 mM in 96% ethanol. The Src family kinase inhibitor pyrazolopyrimidin-2 (PP2) was from Calbiochem (EMD Millipore, Billerca, MA) and was dissolved at 10 mM in dimethyl sulfoxide (DMSO), and the FAK inhibitor PF-573,228 (PF) was originally a pyrazolopyrimidine-2 (PP2) from Calbiochem (EMD Millipore, Billerca, MA) and was dissolved at 10 mM in dimethyl sulfoxide (DMSO), and the FAK inhibitor PF-573,228 (PF) was originally a gift from Pfizer (New York) and later obtained from Sigma-Aldrich and was dissolved at 10 mM in DMSO. The antibodies against total FAK, phospho-Tyr576/577-FAK, phospho-Tyr416-Src, p53, phospho-Tyr94-STAT5, phospho-Src19-MLC2, and normal rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA). Vinculin antibody was from Sigma. Antibodies against Cdk1 and cyclin B1 were obtained from BD Biosciences. Histone H3, c-Yes, FYN3, and phospho-Tyr1007/1008-Jak2 antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA), and an antibody against total Src (anti-v-Src) was from Calbiochem. Antibody against p150 was obtained from BD Transduction Laboratories. Antibody against phospho-Tyr861-FAK was obtained from two different sources, BioSource (Life Technologies, Carlsbad, CA) and Santa Cruz Biotechnology, respectively. Antibodies against phospho-Tyr597-FAK were obtained from both BioSource and Cell Signaling Technology. β-Actin antibody, goat anti-mouse IgG, and goat anti-rabbit IgG were from Sigma-Aldrich. Secondary antibodies used for Western blotting were alkaline phosphatase-conjugated, while secondary antibodies for immunofluorescence analysis were AlexaFluor488- or AlexaFluor568-conjugated.

Cells and cell culture. NIH3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin-streptomycin (GIBCO) at 37°C, 5% CO2, and 95% humidity. Cultures were passaged every 2–4 days. Only passages 10–30 were used. Unless otherwise indicated, experiments were performed at 37°C.

SDS-PAGE and Western blotting. Cells were grown to 80% confluence in 10-cm Petri dishes, washed in ice-cold phosphate-buffered saline (PBS: 136.89 mM NaCl, 2.68 mM KCl, 8.10 mM Na2HPO4, 1.47 mM KH2PO4, and lysed in 95°C SDS lysis buffer (1% SDS, 1 mM Na3VO4, 10 mM Tris HCl, pH 7.4) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Applied Science). Lysates were homogenized by sonication, followed by removal of cell debris by centrifugation at 20,000 g for 5 min at 4°C. Protein concentration of the cleared total cell lysates was determined using a Bio-Rad DC Protein Assay (Bio-Rad Laboratories) and bovine serum albumin (BSA) as the protein standard. Samples for electrophoresis were prepared in NuPAGE LDS Sample Buffer (Novex by Life Technologies), 50 mM dithiothreitol (DTT), and ddH2O. Samples were boiled for 5 min, and equal amounts of protein were loaded to each well. SDS-PAGE gel electrophoresis was carried out in NuPage chambers with NuPAGE precast Tris-acetate 7% gels or Bis-Tris 10% gels under denaturing and reducing conditions, using a BenchMark Protein Ladder (Novex). Separated proteins were electrotransferred to nitrocellulose membranes. Membranes were stained reversibly with Pon- tamo S Solution (Sigma-Aldrich) to confirm equal loading, and were discarded if loading differences were noted. The membranes were blocked (5% nonfat dry milk in TBST) for 1 h at 37°C, incubated with primary antibodies diluted in blocking buffer overnight at 4°C, washed extensively in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20), and incubated for 1 h with alkaline phosphatase-conjugated secondary antibody in blocking buffer at room temperature. Membranes were washed extensively in TBST and developed using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/ni- troblue tetrazolium (NBT) (KPL, Gaithersburg, MD). Membranes were scanned and protein bands quantified using UN-SCAN-IT gel 6.1 software (Silk Scientific, Orem, UT).

Immunofluorescence analysis. Cells seeded on glass coverslips were washed in isotonic saline solution and allowed to equilibrate in isotonic saline solution for 20 min (37°C). After stimulation as indicated, cells were fixed in 2% paraformaldehyde in Tris-buffered saline (TBS, 150 mM NaCl, 10 mM Tris-HCl, 1 mM MgCl2, 1 mM EGTA, pH 7.3) for 15 min at room temperature, followed by 30 min on ice. Next, cells were washed in TBS, permeabilized for 10 min (0.2% Triton X-100 in TBS), blocked for 30 min at room temperature (5% BSA in TBS), and incubated with primary antibodies diluted in TBS overnight at 4°C. Next morning, the preparations were washed extensively in TBS and incubated for 1 h with AlexaFluor488- or AlexaFluor568-conjugated secondary antibody (Invitrogen, 1:600 in TBS + 1% BSA) in the presence or absence of rhodamine phalloidin to label F-actin. Finally, coverslips were washed extensively in TBS + 0.1% Tween-20 (TBST) and mounted with N-propyl-gallate mounting medium (2% wt/vol in PBS/glycerine). Fluorescence was visualized using a Leica DM-IRB/E microscope with Leica TSC-NT confocal laser scanning unit (Leica, Heidelberg, Germany), using a 40X/1.25 NA plan apochromat objective and the 488 and 568 nm Ar/Kr and UV laser lines. For confocal images, optical slice thickness was 1 μm, and pinhole size was 1 airy disk. Images were collected and frame-averaged using Leica software; and 2) an Olympus BX63 epifluorescence microscope 100X/1.4 NA objective. Essentially no labeling was detectable in the absence of primary antibody (not shown). Images were processed in Adobe Photoshop (Brightness/contrast adjustment only and identical for all images from a given dataset).

86Rb+ influx measurements. The procedure was essentially as previously described for taurine influx (17). Briefly, NIH3T3 cells grown to 80% confluence in six-well polyethylene dishes (9.6 cm2 per well) were preincubated (37°C) with the specific inhibitors or vehicle as indicated. All dishes were washed three times by gentle aspiration/ addition of 1 ml isotonic saline solution, followed by addition of 600 μl hypertonic saline solution (37°C). This was followed by exposure

C102 CELL SHRINKAGE ACTIVATES FAK AND Src

AJP-Cell Physiol • doi:10.1152/ajpcell.00070.2014 • www.ajpcell.org

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of the cells in wells 1–5 to 50 μl 86-Rubidium (86Rb⁺, 2,000 Bq/μl) for 0, 1, 2, 3, 4, and 4.5 min, respectively. 86Rb⁺ uptake was terminated by aspiration of the extracellular medium and two washes with 1 ml ice-cold hypertonic saline solution with 30 μM bumetanide followed by cell lysis with 200 μl ethanol (96%). The ethanol was blown off and the cellular 86Rb⁺ was extracted by addition of 600 μl distilled water (20 min), which was transferred to a scintillation vial for estimation of 86Rb⁺ activity (Ultima Gold, Perkin-Elmer). The wells were washed twice with ddH₂O and the total 86Rb⁺ (cpm) at a given time point was estimated as the sum of 86Rb⁺ activity in cell extract plus the two later wash-outs. The cells were dissolved in 100 μl 2 M NaOH and left overnight. Protein content per well was measured by the Lowry method, using BSA as a standard. Counts per well were divided by the protein content (mg protein per well). The 86Rb⁺ influx was estimated from the slope of the 86Rb⁺ content plotted vs. time, and the K⁺ influx was calculated from the ratio between K⁺ and 86Rb⁺ in the extracellular medium as previously described (18).

Immunoprecipitation. Cells were grown to 95% confluence in 14.5-cm Petri dishes, washed three times in standard isotonic saline solution and treated with either isotonic or hypertonic saline solution for 10 min at 37°C. Immediately hereafter, cells were washed in ice-cold PBS, and lysed using NP-40 lysis buffer (1 mM NaF, 1% NP-40, 3 mM Na₃VO₄) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Applied Science) and PhosSTOP EASYpack Phosphatase Inhibitor Cocktail (Roche Applied Science). Lysates were incubated on ice for 1 h, homogenized by sonication, followed by removal of cell debris by centrifugation at 12,000 g for 10 min at 4°C. The protein concentrations of the cleared total cell lysates were determined using a Bio-Rad DC Protein Assay (Bio-Rad Laboratories) and BSA as the protein standard. Samples for input were prepared for electrophoresis using NuPAGE LDS Sample Buffer (Novex by Life Technologies), 250 mM DTT, and ddH₂O. Equal concentrations of protein were precleared in protein G Sepharose 4B conjugate beads (Invitrogen) with gentle rolling for 1 h at 4°C followed by centrifugation at 2,000 g for 2 min at 4°C. Supernatants were transferred to new Eppendorf tubes and incubated with gentle rolling overnight at 4°C with FYN3 (1 μg), c-Yes (2 μg), and v-Src (1 μg), using CdK1 (1 μg) or cyclin B1 (1 μg) and normal rabbit IgG (1–2 μg) as IgG-mouse and IgG-rabbit controls, respectively. Hereafter, samples were incubated with protein G Sepharose 4B conjugate beads or protein G Sepharose 4 fast flow (GE Health Care) for 3 h at 4°C with gentle rolling, succeeded by 4 times wash of beads using NP-40 lysis buffer and in between centrifugation steps at 2,000 g for 2 min at 4°C. Samples were aspirated and prepared for electrophoresis in NuPAGE LDS Sample Buffer (Novex by Life Technologies), 250 mM DTT, and ddH₂O. Samples were boiled for 5 min, centrifuged for 4 min at 15,000 g, and supernatants were transferred to fresh tubes. Twenty microliter samples and equal protein amounts of input sample were loaded onto NuPAGE precast Bis-Tris 10% gels in Novex chambers, and SDS-PAGE gel electrophoresis was carried out under denaturing and reducing conditions, using a BenchMark Protein Ladder (Novex). Subsequent steps were performed as described above.

Statistical analysis. Significance was evaluated using one-way ANOVA with Tukey-Kramer multiple comparisons post test (Fig. 4, 7, A–D and F, and Fig. 8) and two-tailed, paired Student’s t-test (all other figures) with P < 0.05 taken to indicate a statistically significant difference. Data are shown as mean with standard error of the mean (SE) error bars, or are representative of at least 3 independent experiments.

RESULTS

Hyperosmotic stress induces phosphorylation of FAK on Tyr397, Tyr576/577, and Tyr861. To establish the effect of hyperosmotic stress on the phosphorylation of tyrosine residues on FAK in NIH3T3 cells, NIH3T3 cells were exposed to hypertonic saline (575 mOsm compared with 300 mOsm under isotonic conditions) for 2–60 min, as indicated. Lysates were immunoblotted for FAK phosphorylated on the NH₂-terminal Tyr397, the two central sites in the catalytic domain Tyr576/577, or the COOH-terminal Tyr861, respectively. FAK phosphorylation was strongly and rapidly increased on all three phosphorylation sites after hypertonic stress (Fig. 1). Increased phosphorylation was observed already 2 min after hypertonic exposure, with the highest increase observed for pTyr397. Maximal phosphorylation on all three sites was observed after 10 min. After 60 min the phosphorylation of Tyr397 and Tyr576/577 was no longer significantly different from the isotonic level, while the Tyr861 phosphorylation was sustained.

Exposure of the cells to hypotonic saline (175 mOsm) to induce cell swelling also affected the phosphorylation of FAK, although to a lesser extent than the hypertonic saline. Thus we observed a rapid, significant increase (~40%) in phosphorylation of Tyr861 after 2 min, and a significant decrease (~25%) in phosphorylation of Tyr397 after 60 min (n = 3, data not shown). The effect of osmotic swelling on FAK phosphorylation was not further pursued here, but this result substantiates the notion that FAK phosphorylation is sensitive to osmotic volume perturbations.

Fig. 1. Effect of hypertonic stress on the phosphorylation of FAK on Tyr397, Tyr576/577, and Tyr861. Western blot analysis of cells stimulated with isotonic (300 mOsm; I) or hypertonic (575 mOsm; H) saline solution for 2, 10, 20, 30, or 60 min. Protein phosphorylation levels were determined using antibodies specific for FAK phosphorylated on Tyr397, Tyr576/577, and Tyr861, respectively. Total FAK was used as loading control. Quantification of the blots is shown as means with SE error bars relative to the isotonic sample at the corresponding time point. *P < 0.05, **P < 0.01, ***P < 0.001. The no. (n) of independent experiments per condition was 4–6.
Subcellular localization of pFAK and vinculin under hyperosmotic stress. To investigate whether activated FAK localizes to FAs in NIH3T3 cells undergoing osmotic shrinkage, the subcellular localization of pTyr397-FAK and pTyr576/577-FAK was examined by immunofluorescence analysis, using vinculin as a marker for focal adhesions (16). pTyr397-FAK was essentially undetectable under isotonic conditions (Fig. 2A, left panel). Osmotic cell shrinkage (575 mOsm, 10 min) increased the number of vinculin puncta, suggesting an increased number of FAs under these conditions (Fig. 2A and B, right panel). In agreement with the immunoblot data, osmotic shrinkage increased the phosphorylation of both Tyr397 and Tyr576/577, although the latter was also substantially phosphorylated under isotonic conditions. Finally, pTyr397-FAK (Fig. 2A) and Tyr576/577-FAK (Fig. 2B) colocalized with vinculin puncta, indicative of localization in FAs (Fig. 2B, left panel).

Src family kinases are activated by hypertonic stress and localize to FAs. Next, we investigated the effect of osmotic shrinkage on Src family kinase activation in NIH3T3 cells. Osmotic cell shrinkage was induced by hypertonic exposure (575 mOsm) for 2–60 min, as indicated, and lysates were immunoblotted for Src family kinases phosphorylated on Tyr416 in the catalytic domain. Tyr416 phosphorylation was significantly increased 10 min after hypertonic exposure, and continued to increase up to 60 min after stimulation, at which time Src phosphorylation was almost threefold higher than under isotonic conditions (Fig. 3A). Under isotonic conditions, pTyr416-Src was detectable near FAs, as indicated by colocalization with vinculin (Fig. 3B, left panel). Ten minutes after hypertonic exposure pTyr416-Src staining was markedly increased and colocalized with that of vinculin at FAs (Fig. 3B, right panel).

Src inhibition strongly inhibits shrinkage-induced Tyr576/577 and Tyr861 phosphorylation of FAK. To investigate the role of Src kinase activity in shrinkage-induced FAK phosphorylation, the Src kinases were inhibited by pyrazolopyrimidine-2 (PP2, 10 μM) (13), for 10 min prior to, and during, isotonic (300 mOsm) or hypertonic (575 mOsm) exposure. PP2 treatment attenuated phosphorylation of FAK on Tyr576/577 and Tyr861 under both iso- and hypertonic conditions, while phosphorylation at Tyr397 was unaffected (Fig. 4). Inhibition with PP2 for 20 min gave similar results (data not shown). These results indicate that Src kinases contribute to both basal and shrinkage-induced phosphorylation of FAK at Tyr576/577 and Tyr861, but not at Tyr397.

Src plays a major role in the shrinkage-activation ofNKCC1. To assess the possible role of Src kinases in shrinkage-induced NKCC1 activation, we used the radioactive isotope 86-Rubidium (86Rb⁺) as a K⁺ analog to estimate K⁺ influx induced by hypertonic shrinkage (575 mOsm), in the presence or absence of the NKCC1 inhibitor bumetanide (30 μM), for 0–4 min. Consistent with the major role of NKCC1 in this process, bumetanide inhibited shrinkage-induced K⁺ influx by more than 50% (Fig. 5A). The majority of the bumetanide-insensitive K⁺ influx is most likely via Na⁺/K⁺-ATPase, as previously shown in Ehrlich cells (18). PP2 (10 μM, present from 10 min prior to the hypertonic challenge and throughout the experiment) inhibited the bumetanide-sensitive fraction of the K⁺ influx by ~50% (Fig. 5, B and C), indicating a role for Src in shrinkage-activation of NKCC1.

Src, but not Fyn or Yes, is phosphorylated at Tyr416 upon osmotic shrinkage of NIH3T3 cells. The pTyr416 antibody employed recognizes all Src kinase isoforms phosphorylated on this autophosphorylated tyrosine residue. To determine which of the most widely expressed Src isoforms—Src, Fyn, and Yes—was phosphorylated during osmotic shrinkage of
NIH3T3 cells, we therefore immunoprecipitated each of these kinases, followed by immunoblotting for phosphorylation at Tyr416 (Fig. 6). Fyn, Src, and Yes were all immunoprecipitated from NIH3T3 cells under iso- and hypertonic conditions (Fig. 6, A–C); however, only Src exhibited increased phosphorylation at Tyr416 upon hypertonic stress (Fig. 6, D–F). Thus these findings indicate that in NIH3T3 cells, Src is phosphorylated during osmotic shrinkage, whereas Fyn and Yes are not.

FAK contributes modestly to the shrinkage-activation of NKCC1. To examine the role of FAK in shrinkage-activation of NKCC1, the FAK inhibitor PF-573,228 (PF) was employed. Cells were incubated with or without 10 μM PF for 1 h, followed by iso- or hypertonic exposure in the continued presence of PF. Treatment with PF significantly inhibited hypertonic stress-induced phosphorylation of FAK at Tyr397, whereas phosphorylation at Tyr576/577 and Tyr861 was not significantly inhibited by PF (Fig. 7, A–C). In contrast, phosphorylation of Src at Tyr416 was increased by treatment with PF, and this was significant after 10 min hypertonic stress (Fig. 7D). The mechanism remains to be elucidated, but the results strongly indicate that the shrinkage-induced activation of Src is not downstream from FAK. Shrinkage-induced NKCC1 activation was modestly, albeit significantly, inhibited by PF (Fig. 7E), suggesting that FAK plays a minor role in shrinkage-activation of NKCC1. Finally, because FAK is an important regulator of cell death/survival balance, we evaluated the potential involvement of FAK in the shrinkage-induced activation of p53. NIH3T3 cells were preincubated with DMEM in the presence or absence of PF (10 μM) and treated with iso-

Fig. 3. Effect of hypertonic stress on the phosphorylation of Src on Tyr416 in NIH3T3 cells. A: Western blot analysis of cells stimulated with isotonic (300 mOsm) or hypertonic (575 mOsm) saline solution for 2, 10, 20, 30, or 60 min. Src activity was determined using an antibody specific for Src phosphorylated on Tyr416. Total Src was used as loading control. Quantification of the blots is shown as means with SE error bars relative to the isotonic sample at the corresponding time point. **P < 0.01, ***P < 0.001. The no. (n) of independent experiments was 5. Representative Western blots are shown at right. B: cells grown on coverslips were treated with isotonic (300 mOsm) or hypertonic (575 mOsm) saline solution for 10 min, followed by paraformaldehyde fixation and immunofluorescence analysis. Src phosphorylated on Tyr416 (green) and vinculin (red) were detected with anti-pTyr416-Src and anti-vinculin antibodies, as shown. Scale bar, 10 μm; insets, 5 μm. Images are representative of 3 independent experiments.

Fig. 4. Effect of the Src family kinase inhibitor PP2 on the hypertonic stress induced phosphorylation of FAK in NIH3T3 cells. Western blot analysis of cells preincubated with cell culture medium ± 10 μM pyrazolopyrimidine-2 (PP2) for 30 min and subsequently treated with isotonic (300 mOsm) or hypertonic (575 mOsm) saline solution for 10 min in the presence (+) or absence (−) of PP2 (10 μM). Protein phosphorylation levels were determined using antibodies specific for FAK phosphorylated on Tyr397, Tyr576/577, and Tyr861, respectively, and for Src phosphorylated on Tyr416. Total FAK and total Src were used as loading controls. Data are shown as means with SE error bars relative to the isotonic control. *P < 0.05, **P < 0.01, ***P < 0.001, ns, no significance. The no. (n) of independent experiments per condition was 4.
Fig. 5. Effect of inhibition of NKCC1 or Src on $^{86}$Rb$^+$ influx in NIH3T3 cells under hypertonic stress. A: $^{86}$Rb$^+$ influx. The shrinkage-activated K$^+$ influx was estimated using the radioactive isotope $^{86}$rubidium ($^{86}$Rb$^+$) as a K$^+$ analog. Cells were treated with hypertonic (575 mOsm) saline solution in the presence or absence of 30 μM of the NKCC1 inhibitor bumetanide, and the cells were exposed to $^{86}$Rb$^+$ for 0–4 min. $^{86}$Rb$^+$ counts per minute (cpm) was normalized to the protein concentration (cpm/mg protein). The no. (n) of independent experiments was 9. B and C: effect of theSrc inhibitor PP2 on the bumetanide sensitive K$^+$ influx in NIH3T3 cells under hypertonic stress. Cells were treated with hypertonic (575 mOsm) saline solution in the presence or absence of 30 μM bumetanide and/or 10 μM PP2, and the cells were exposed to $^{86}$Rb$^+$ for 0–4 min. Cells treated with bumetanide were preincubated with 30 μM bumetanide for 10 min, while cells treated with PP2 were preincubated with 10 μM PP2 for 30 min before the experiment. $^{86}$Rb$^+$ cpm was normalized to the protein concentration (cpm/mg protein). The no. (n) of independent experiments was 7–9. C: the values are calculated from the difference in slopes without PP2 in A (Ctrl) and with PP2 in C (PP2) and the outer specific activity of K$^+$, where $^{86}$Rb$^+$ is used as a measure for K$^+$. **P < 0.01.

(300 mOsm) or hypertonic saline (575 mOsm) for 4.5 h in the continued presence/absence of PF. As previously shown, osmotic cell shrinkage strongly increased the expression of p53 compared with that in isotonic control cells, yet this increase was unaffected by PF (Fig. 7F).

Jak2, but not MLC, is phosphorylated upon osmotic shrinkage, in a Src-dependent manner. We next asked whether Jak2 and MLC were phosphorylated upon osmotic shrinkage of NIH3T3 cells, and whether their phosphorylation was Src dependent. NIH3T3 cells were preincubated for 30 min in the presence or absence of PP2 (10 μM) and subsequently exposed to iso- (300 mOsm) or hypertonic (575 mOsm) saline for 2 or 10 min in the continued presence or absence of PP2. Treatment with PP2 significantly inhibited shrinkage-induced Jak2 activation, both after 2 and 10 min (Fig. 8, A and B). In contrast, STAT5 phosphorylation was independent of osmotic cell shrinkage and PP2 treatment (Fig. 8, A and B), indicating that Jak2 may not act via the Jak2/STAT5 pathway during cell shrinkage in NIH3T3 cells. Surprisingly, MLC activation was similarly insensitive to osmotic cell shrinkage and PP2 treatment (Fig. 8, A and C), suggesting that cell shrinkage regulation in NIH3T3 cells is independent of MLCK.
Fig. 7. Effect of the FAK inhibitor PF-573,228 on FAK and Src phosphorylation, the bumetanide-sensitive 86Rb+ influx, and p53 expression in NIH3T3 cells under hypertonic stress. A–D: Western blot analysis of cells preincubated with cell culture medium ± 10 μM PF-573,228 (PF) for 1 h and subsequently treated with isotonic (300 mOsm) or hypertonic (575 mOsm) saline solution in the presence or absence of 10 μM PF for 1, 5, or 10 min. Protein phosphorylation levels were determined using antibodies specific for FAK phosphorylated on Tyr397 (A), Tyr576/577 (B), and Tyr861 (C), and for Src phosphorylated on Tyr416 (D). Total FAK and total Src were used as loading controls. Data are shown as means with SE error bars relative to the isotonic control. The no. (n) of independent experiments was 3–4. E: bumetanide-sensitive 86Rb+ influx ± 10 μM PF. Cells were treated with hypertonic (575 mOsm) saline solution for 10 min in the presence or absence of 30 μM bumetanide and/or 10 μM PF, and the cells were exposed to 86Rb+ for 2–10 min. Cells treated with bumetanide were preincubated with 30 μM bumetanide for 10 min, while cells treated with PF were preincubated with 10 μM PF for 1 h before the experiment. Data are obtained from the slope of 86Rb+ influx curves and shown as means with SE error bars. The no. (n) of independent experiments was 5. F: Western blot analysis of the protein expression of p53 in cells preincubated with cell culture medium ± 10 μM PF for 1 h and subsequently treated with isotonic (300 mOsm) or hypertonic (575 mOsm) saline solution ± 10 μM PF for 4.5 h. p53 protein expression was determined using a specific anti-p53 antibody. Histone H3 was used as a loading control. Data are shown as means with SE error bars relative to the isotonic control. The no. (n) of independent experiments was 5. *P < 0.05, **P < 0.01, ***P < 0.001. ns, no significance.

**DISCUSSION**

**Hypertonic shrinkage rapidly activates FAK, Src, and Jak2 in NIH3T3 cells.** We show here that osmotic cell shrinkage by exposure of NIH3T3 cells to hypertonic (575 mOsm) saline induced a rapid, transient increase in FAK phosphorylation at Tyr397 and Tyr576/577, while phosphorylation of Tyr861 was slower and of longer duration. Phosphorylation of Src family kinases at Tyr416 was also strongly increased by hypertonic exposure, and this could be ascribed to phosphorylation of the Src isoform, whereas shrinkage-induced phosphorylation of Fyn and Yes could not be detected. While the specific Src isoform activated by hypertonic stress appears to be cell-type specific, this agrees well with previous findings suggesting that Src kinases play a central role in volume sensing and are activated in response to cell shrinkage (20, 23, 36). Both pTyr397- and pTyr576/577-FAK and pTyr416-Src localized partially to FAs in osmotically shrunken cells. FAK, when phosphorylated on Tyr397, recruits Src to its SH2 and SH3 domains, followed by Src-mediated phosphorylation of FAK at additional sites (32). In accordance with this sequence of

**AJP-Cell Physiol • doi:10.1152/ajpcell.00070.2014 • www.ajpcell.org**
events, shrinkage-induced phosphorylation of FAK at Tyr576/577 and Tyr861, yet not at Tyr397, was abolished by the Src inhibitor PP2. This supports previous work reporting PP2-induced inhibition of shrinkage-induced FAK phosphorylation on Tyr576 and Tyr861 in Swiss 3T3 cells (26). At 10 μM, PP2 may also inhibit several Ser/Thr kinases, including p38, ERK2, and PKC, albeit with lower affinity than for Src (3). However, several lines of evidence support that the observed effect of PP2 reflects a role for Src in FAK phosphorylation. Thus shrinkage-induced Tyr861-FAK phosphorylation was abolished in cells lacking Src expression (26), and a chemically different Src inhibitor, AZD0530, inhibited phosphorylation of FAK in human breast cancer cells (19).

The fast, PP2-insensitive Tyr397-phosphorylation of FAK is consistent with, albeit obviously not unique to, the idea that cell shrinkage induces integrin clustering, leading to recruitment and Tyr397-autophosphorylation of FAK. As discussed in Ref. 15, integrins have been widely proposed to serve as cell volume sensors. In hepatocytes (14) and in cardiac myocytes (37), integrins have been identified as a major osmosensory

Fig. 8. Effect of the Src family kinase inhibitor PP2 on the hypertonic stress-induced phosphorylation of Src, JAK2, STAT5, and MLC2 in NIH3T3 cells. Western blot analysis of cells preincubated with cell culture medium ± 10 μM PP2 for 30 min and subsequently treated with isotonic (300 mOsm) or hypertonic (575 mOsm) saline solution for 10 min in the presence (+) or absence (−) of PP2 (10 μM). A: representative Western blots. B: quantification of pJAK2 and pSTAT5 levels at 2 min (top) and 10 min (bottom) after cell shrinkage. C: quantification of pMLC levels 2 (left) and 10 min (right) after cell shrinkage. Protein phosphorylation levels were determined using antibodies specific for pTyr1007/1008-JAK2, pTyr694-STAT5, and p-Ser19-MLC2, respectively. β-Actin was used as loading control. Data are shown as means with SE error bars relative to the isotonic control. *P < 0.05, **P < 0.01, ***P < 0.001. The no. (n) of independent experiments per condition was 3.

Fig. 9. Model: osmotic cell shrinkage induces integrin clustering, followed by recruitment of FAK to focal adhesions, and its autophosphorylation on Tyr397. Src is also activated by osmotic stress, in a manner independent of FAK, and Src phosphorylates FAK on Tyr576/577 in the catalytic domain and on the NH2-terminal Tyr861. Activated Src also contributes to the activation of Jak2. Jak2, and to a limited degree also FAK, in turn contributes to the activation of NKCC1. Our findings suggest that additional mechanisms contribute to the shrinkage-induced activation of NKCC1. Black arrows indicate pathways found to be involved in the present study, while gray arrows represent suggested pathways.
system, and in both cell types this was associated with activation of FAK. It may be noted that integrins are highly sensitive to local intra- and extracellular pH (34, 41), which is likely to be altered upon cell shrinkage due to the shrinkage-induced activation of the integrin-associated Na+/K+ exchanger (NHE1), which is known to increase pH_i locally around FAs (31). While not experimentally addressed here, this would potentially create a signaling loop in which NHE1 activity could reinforce that of the other major shrinkage-activated osmolyte transporter, NKCC1.

To obtain further insight into the signaling events elicited by osmotic shrinkage in NIH3T3 cells and relevant to NKCC1 activation, we assessed the effect of hypertonic exposure on Jak2 and MLC phosphorylation and found that while Jak2 was phosphorylated rapidly by osmotic shrinkage, MLC was not. Interestingly, Jak2 phosphorylation was strongly inhibited by PP2, in agreement with previous reports that in some contexts, Jak2 activation is Src-dependent (1).

Inhibition of Src inhibits Jak2 phosphorylation and shrinkage-induced NKCC1 activation. About 50% of the shrinkage-induced NKCC1 activity in NIH3T3 cells was prevented by PP2, indicating a major role of Src family kinases in this process. It has previously been shown that PP2 inhibits Na+/K+-ATPase activity in eye lens cultures from rabbits, probably through inhibition of Src (42). In our study, the hypertonically stimulated K+ influx was not reduced further by simultaneous bumetanide and PP2 treatment compared with PP2 alone, suggesting that bumetanide and PP2 both inhibit the K+ influx via NKCC1. PP2 also strongly inhibited the shrinkage-induced phosphorylation of FAK on Tyr577 and Tyr861 in NIH3T3 cells. However, inhibition of FAK only marginally decreased the shrinkage-induced NKCC1 activity, suggesting that in these cells, FAK plays at most a minor role in shrinkage activation of NKCC1. We have previously shown that hyperosmotic stress elicits p53 phosphorylation and increases p53 expression in NIH3T3 cells (9). FAK has been shown to favor MDM2-dependent ubiquitination and degradation of p53 (25); hence, we hypothesized that FAK inhibition might increase the p53 protein level after osmotic shrinkage. However, shrinkage-induced induction of p53 protein expression in NIH3T3 cells was unaffected by inhibition of FAK, arguing against a role for FAK in this process in NIH3T3 cells.

Jak2 has been assigned a role in NKCC1 activation by prolactin, in a manner involving tyrosine phosphorylation of NKCC1 (39), and has been shown to be activated by osmotic shrinkage in several cell types (10, 11). Furthermore, while the mechanism is not fully elucidated, Src has been implicated in Jak2 activation (2). We report here that in NIH3T3 cells, Jak2 was rapidly activated upon hypertonic shrinkage and this effect was strongly inhibited by PP2. In contrast, phosphorylation of MLC, the only known substrate for MLCK, was unaffected by both shrinkage and PP2. Taken together, hence, our data are consistent with the hypothesis that the effect of PP2 could be mediated via Jak2, whereas they do not support a role for MLCK activity and MLC phosphorylation in NKCC1 activation in NIH3T3 cells. This in fact agrees well with conclusions from studies in kidney epithelial cells, arguing against a role for the MLCK-pMLC axis in shrinkage-induced NKCC1 activation (5, 6), whereas they seem to be in variance with studies from some other cell types (for a discussion, see Ref. 15).

A working model based on our findings is shown in Fig. 9. The data are consistent with the interpretation that in NIH3T3 cells, osmotic shrinkage elicits integrin clustering, followed by recruitment of FAK to FAs, and its autophosphorylation on Tyr397. Src (but not Fyn or Yes) is also activated by osmotic stress, in a manner independent of, or even attenuated by, FAK (as PF augments, rather than inhibits, Src activation). Src phosphorylates FAK on Tyr576/577 and Tyr861, and contributes to the activation of Jak2. Jak2, and to a limited degree also FAK, in turn contributes to activation of NKCC1. Given that only 50% of the NKCC1 activity could be blocked by PP2, our findings suggest that additional mechanisms contribute to the shrinkage-activation of NKCC1.

In conclusion, we show here that Src, FAK, and Jak2 are rapidly activated by osmotic shrinkage of NIH3T3 fibroblasts, and that Src-dependent signaling, possibly through Jak2, contributes substantially to shrinkage-activation of NKCC1.

ACKNOWLEDGMENTS
The technical skills of B. J. Hansen and K. F. Mark (Univ. of Copenhagen) are gratefully acknowledged.

GRANTS
This work was supported by The Danish Council for Independent Research/ Natural Sciences (Grants 09-064182 and 10-085373) and by The Lundbeck Foundation and the Bdr. Hartmann Foundation.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
Cell shrinkage activates FAK and Src


