SRC REGULATES DISTINCT PATHWAYS FOR CELL VOLUME CONTROL THROUGH VAV AND PHOSPHOLIPASE C GAMMA

Elisabeth T. Barford†, Ann L. Moore*, Richard F. Melnick*, and Steven D. Lidofsky*†
Departments of *Medicine and †Pharmacology, University of Vermont

Running Title: Src and Cell Volume Regulation

Address correspondence to: Steven Lidofsky, M.D., Ph.D., Burgess 414 MFU, University of Vermont, Burlington VT 05401, Phone: 802-847-5990, FAX: 802-847-4928, E-mail: steven.lidofsky@uvm.edu

Cell volume recovery in response to swelling requires reorganization of the cytoskeleton and fluid efflux. We have previously shown that electrolyte and fluid efflux via K⁺ and Cl⁻ channels is controlled by swelling-induced activation of phospholipase Cγ (PLCγ). Recently, integrin engagement has been suggested to trigger responses to swelling through activation of Rho family GTPases and of Src kinases. Since both PLCγ and Rho GTPases can be regulated by Src during integrin-mediated cytoskeletal reorganization, we sought to identify swelling-induced Src effectors. Upon hypotonic challenge, Src was rapidly activated in transient plasma membrane protrusions, where it colocalized with Vav, an activator of Rho GTPases. Inhibition of Src with PP2 attenuated phosphorylation of Vav. PP2 also attenuated phosphorylation of PLCγ, and inhibited swelling-mediated activation of K⁺ and Cl⁻ channels and cell volume recovery. These findings suggest that swelling-induced Src regulates cytoskeletal dynamics, through Vav, and fluid efflux, through PLCγ, and thus can coordinate structural reorganization with fluid balance to maintain cellular integrity.

Cells swell in response to a variety of stimuli and must restore their volume in the continued presence of osmotic challenge to survive. Such stimuli exert mechanical strain on the structural components of the cell through increased fluid pressure, which must be diminished to maintain cellular integrity. In many cell types, restoration of volume is achieved by intracellular Ca²⁺ mobilization, which triggers fluid and electrolyte efflux through plasma membrane ion channels (1). Previous work in our laboratory has shown that the Ca²⁺ increases necessary to open these ion channels occur through tyrosine phosphorylation and activation of phospholipase Cγ (PLCγ) (2,3). The identity of the responsible tyrosine kinase is unknown.

In addition to the restoration of fluid balance, a swollen cell must reorganize its disrupted cytoskeleton. Recent observations suggest that this is achieved by signaling through integrins (4,5). In response to swelling, the Rho family GTPases, Cdc42 and Rac1, are activated to form membrane protrusions identical to those seen following integrin engagement (4). Src tyrosine kinases have also been shown to be activated upon cell swelling via a mechanism consistent with integrin dependence, and kinase activity appears to be required for volume recovery (5). Taken together, these findings suggest that integrins are involved in transducing osmotic signals to initiate cell volume recovery through the activation of Src (5) and Cdc42 and Rac1 (4).

Integrins transduce signals from many types of mechanical stimuli distinct from osmotic stress, and these can lead to activation of Src family kinases. Integrins also function as mechanosensors during cell migration, and similar downstream effectors, including Src and Rho GTPases, are often activated by mechanotransduction and cell motility (6). In mechanotransduction, integrins activate Src independently of whether the force initiates from outside or from within the cell (7). If integrin activation of Src during volume control is similar to integrin activation of Src through mechanotransduction and migration, the regulation of downstream pathways may share similarities.

In cell migration, Rho GTPases regulate actin cytoskeletal dynamics through diverse activators and effectors (8). The functions of the Rho GTPases, Cdc42, Rac1 and RhoA, must be spatially and temporally balanced in order for productive motility to occur (9). Cdc42 and Rac1 help to initiate cell migration via mediators that

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trigger the formation of an actin polymerization complex at the leading edge, where localized actin polymerization provides the force necessary to form a membrane protrusion (10-12). Src controls both the activation of Cdc42 and Rac1 (13) and the inhibition of RhoA (14) at the leading edge. Behind the leading edge, RhoA is reactivated (15) to form stress fibers to mediate contractile forces requisite for cell motility (16), and it then recruits Src (17) to regulate the formation of focal adhesions (18). At the rear of the cell, RhoA mediates disassembly of focal adhesions in a Src-dependent fashion (19). Thus, through the coordinate activation and inhibition of Rho GTPases, Src acts to control the spatial and temporal action of cytoskeletal regulators required for optimal cell migration (20).

A variety of activators for Rho family members, guanine nucleotide exchange factors (GEFs), can interact with Cdc42 and/or Rac1 (21), and a number of these GEFs have been implicated in cell spreading and migration (13,22-25). One of these, Vav, is known to be activated by Src (26), and such activation has been shown to be dependent on integrin engagement during cell spreading (27). In particular, a Src-Vav2 complex has been shown to regulate Rac activation and Rho inactivation during growth factor-stimulated cell migration (28). In contrast to the GEFs DOCK180 and Tiam1, Vav can activate Cdc42, in addition to Rac1 (29), and Vav2 is ubiquitously expressed (30). We therefore sought to determine whether Vav was linked to swelling-mediated protrusion formation, and if so, whether it was activated by Src.

Cell volume regulation is critical to maintenance of normal organ level function in liver, which is subjected to osmotic stresses that result from the uptake and metabolism of nutrients absorbed from the gut after each meal (31,32). Recovery from hepatocellular swelling requires fluid and electrolyte efflux through specific activation of K⁺ and Cl⁻ channels (33). We have found that this process is triggered by stimulation of PLCγ, generation of inositol 1,4,5-trisphosphate (IP₃), and release of Ca²⁺ from internal stores via activation of IP₃ receptors (2,3). Intriguingly, PLCγ activity has been shown to be essential for cell migration (34), and PLCγ has been shown to be activated by tyrosine phosphorylation in response to mechanical strain (35). Therefore, we sought to determine whether volume-sensitive activation of PLCγ, and by extension, activation of volume-sensitive ion channels were dependent on Src activity.

In this work, we investigated whether in response to cell swelling, Src could function to coordinate dynamic changes in the actin cytoskeleton and the opening of volume-sensitive ion channels by activation of different effectors within the cell. Our findings suggest that through the recruitment and activation of Vav and PLCγ, Src coordinates the reorganization of the actin cytoskeleton with activation of ion channels to promote cell volume recovery.

**EXPERIMENTAL PROCEDURES**

**Reagents and antibodies** — The acetoxymethyl ester (AM) of calcein and FITC-, Alexa 488-, Alexa 568- conjugated anti-rabbit and anti-mouse antibodies, and Alexa 647-conjugated phalloidin were purchased from Molecular Probes. Activated phosphotyrosine-specific PLCγ1 (Tyr-783) and phosphotyrosine-specific Src family (Tyr-416) rabbit antibodies were obtained from Cell Signaling. Monoclonal phosphotyrosine antibody (clone 4G10) as well as its agarose conjugate, monoclonal phosphotyrosine-specific Src family (Tyr-416) antibody (clone 9A6), and monoclonal pp60Src antibody (clone GD11), as well as the GD11-agarose conjugate, were purchased from Upstate Biotechnology. Monoclonal mouse anti-FAK antibody was from Transduction Laboratories. Activated phosphotyrosine-specific FAK (Tyr-397) rabbit antibody was purchased from Biosource International. Vav-2 goat antibody, activated phosphotyrosine-specific Vav-2 (Tyr-172) rabbit antibody, actin goat antibody, and donkey anti-goat HRP secondary antibody were obtained from Santa Cruz Biotechnology. (We assume that the phosphotyrosine-specific Vav antibody may cross-react with tyrosine-phosphorylated Vav1 and tyrosine-phosphorylated Vav3 based on the properties of a similarly produced antibody (36) and after consultation with Santa Cruz Biotechnology about the antibody purification). HRP-, CY3- and CY5-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratory. PP2 (4-amino-5-(4-chlorophenyl)-7-
Cells and cell culture — HTC rat hepatoma cells were cultured as previously described (37). Except where noted, cells were placed in an isotonic (approximately 300 mOsm) standard extracellular solution (SES). SES contained (in mM): 140 NaCl, 4 KCl, 1 CaCl2, 2 MgCl2, 1 KH2PO4, 10 glucose, and 10 HEPES (pH 7.4). Cell swelling was produced by exposure to a hypotonic solution. Unless specified, the hypotonic solution (HES, approximately 240 mOsm) was identical to SES, except that the concentration of NaCl was 84 mM. All experiments were performed at room temperature except where noted. For some experiments, cells were transfected with a pcDNA plasmid encoding mouse c-Src (a generous gift of Dr. Anthony Morielli) using Superfect (Qiagen) according to the manufacturer’s protocols.

Live cell imaging and cell volume analysis — Three-dimensional reconstruction of real time high-resolution images of cells undergoing hypotonic challenge were performed as follows. Cells plated on coverslips were incubated with calcine-AM (5 µM) in SES. Cells were then placed in a perfusion chamber housed on the stage of a DeltaVision Restoration Olympus IX70 microscope and imaged at 19 °C, with a 40× oil lens, NA 1.35 and a Coolsnap camera (HQ Photometrics). The fluorescence excitation and emission wavelengths were 488 and 530 nm, respectively. Time lapse data were collected using twenty 1 µm steps (with each full z-series acquired at 1 min intervals). In the fifth minute, the continuous SES perfusion was changed to HES and the experiments continued for a total of 30 min. Data collection, deconvolution, and mathematical analysis of cell volume were made using DeltaVision software softWoRx Ver. 3.2 (Applied Precision). Deconvolution proceeded by the standard algorithm, and the three-dimensional visualization by the quick projection option. The mathematical analysis proceeded by careful thresholding of the signal such that two-dimensional polygons of each cell slice were defined in each z section. The validity of these was confirmed by visual inspection of each z section. The volume calculations built three-dimensional polygons from these thresholded images. Images were minimally processed using Adobe Photoshop V7 software.

Alterations in cell size were also determined by measurement of cross-sectional area, which has been shown to reflect changes in liver cell volume following osmotic challenge (38). Cells on coverslips were incubated with calcine-AM, and placed in a perfusion chamber (Warner Instruments RC-26G) on the stage of an inverted Nikon Diaphot 200 fluorescence microscope. Cell area was determined from fluorescent images taken, using a 40 × oil objective, NA 1.3, by a Hamamatsu Orca-ER C4742-95 digital camera and quantified using MetaMorph Version 4.6r4 integrated morphometry analysis software (Universal Imaging). The fluorescence excitation and emission wavelengths were 488 and 530 nm, respectively. Images were acquired at 30-sec intervals and relative cell area (A) was calculated by dividing the measured area at a given time by the mean area measured under isotonic conditions during the 5 min period prior to hypotonic exposure. The extent of cell volume recovery after swelling (%RVD) was calculated from the equation below, where Amax is the relative maximum cell area after swelling, and A15 is the relative cell area 15 min after hypotonic exposure.

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\%RVD = \left( \frac{A_{\text{max}} - A_{15}}{A_{\text{max}} - 1} \right) \times 100
\]

Immunoprecipitation and immunoblot analysis — Extraction of whole cell lysates and immunoprecipitations were performed as previously described (3). In selected experiments, crude membrane and cytosolic fractions were obtained as follows. Ice cold extraction buffer (20 mM TRIS , pH 7.5, 20 mM p-nitrophenyl phosphate, 1 mM EGTA, 50 mM NaF, 1mM Na orthovanadate, 1 mM phenylmethyl sulfonyl fluoride, with complete protease inhibitor mixture and phosphatase inhibitor) was added to the cells, and the plates were left on ice for 10 min. Cells were then scraped off and homogenized (Dual 20 with Teflon pestle) and then centrifuged for 10 min at 800 × g. Equal volumes of homogenates were then centrifuged at 50,000 × g for 30 min.
The cytosolic supernatant was carefully removed, and the crude membrane pellet was solubilized in the same volume buffer (10 mM TRIS, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM Na orthovanadate, 1% Triton X-100, 0.5% NP-40, 1% Na deoxycholate, 0.1% SDS, protease and phosphatase inhibitors) with vortexing.

Proteins were resolved by SDS electrophoresis on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with appropriate primary antibodies, as recommended by the manufacturer. Proteins were detected by exposure to horseradish peroxidase-conjugated donkey anti-rabbit, anti-mouse, or anti-goat antibody and enhanced chemiluminescence (Super Signal, Pierce). Densitometric analysis was performed using an Alpha Imager 2000 (Alpha Innotech).

**Immunofluorescence and colocalization analysis** — Cells seeded onto glass coverslips were grown overnight, and the culture medium replaced with L15 medium with 10% calf serum at room temperature. To start the experiment, medium was then replaced with either SES (isotonic) or HES (hypotonic). At designated times, cells were fixed with 4% formaldehyde in PHEM buffer (pH 6.1) and permeabilized with 0.2% Triton X-100, 300 mM sucrose in PBS, as previously described (3), or with 0.2% SDS as noted in figure legend. Coverslips were washed, blocked, and incubated with primary antibody and then with fluorescent label-conjugated secondary antibody and fluorescent-labeled phalloidin. Samples were mounted onto slides with Aquamount (Polysciences) and viewed on the DeltaVision Restoration microscope system described above with a 60 × oil lens, NA 1.4. Data were collected using either a Coolsnap camera (HQ Photometrics) or a CH350 CCD camera (Kodak) with different z steps as noted, and deconvolved using the DeltaVision software softWoRx Ver. 3.2 (Applied Precision). Images were processed using Adobe Photoshop V7 software without gamma adjustment of brightness and contrast, except in Figure 4A as noted in the figure legend. Quantification of colocalization was performed on a set of experiments in which there was minimal previewing (to reduce photobleaching). Percent protrusive cells were analyzed by three-dimensional analysis of deconvolved z-series, and positive cells were scored as those with protrusions detected no lower than 1 μm above the base of the cell (to exclude lamellipodia). Overlap of activated Src and activated Vav was calculated using Volocity3 software (Improvision) on deconvolved DeltaVision files.

**Measurement of membrane currents** — Whole cell currents were measured using patch clamp recording techniques as described previously (2). The extracellular solution was SES, and the hypotonic solution was identical to SES, except that the concentration of NaCl was 98 mM. The pipette solution contained (in mM) 10 NaCl, 130 KCl, 0.5 CaCl₂, 2 MgCl₂, 1 EGTA, and 10 HEPES (pH 7.30). Under these conditions, measurements at a holding potential of 0 mV reflect K⁺ currents, and measurements at a holding potential of -80 mV reflect Cl⁻ currents (2).

**Statistics** — All results are presented as means ± SE, where n represents either the number of cells, experiments, or fields of cells. Comparisons were made with the use of unpaired Student’s t test, or Dunnett’s multiple comparison test (ANOVA) as appropriate, and p<0.05 was considered to be significant. Correlation coefficients were determined by nonparametric statistical analysis (Spearman), and significance determined with two-tailed analysis at the 95% confidence level. The Dunnett’s and Spearman analyses were determined using Prism version 3.0cx (GraphPad Software, Inc).

**RESULTS**

**Transient membrane protrusions form in response to cell swelling** — Osmotic stress induces dramatic cell shape changes in a number of cell types (39), including rapid formation of membrane protrusions in fibroblasts upon hypotonic challenge (4). Because we previously had observed irregular cell morphologies in connection with swelling-induced plasma membrane PLCγ localization in HTC hepatoma cells (3), we investigated whether hypotonic exposure of these cells would elicit plasma membrane protrusions analogous to those in fibroblasts, and how the formation of swelling-induced membrane protrusions temporally correlated with volume recovery.
To test this, we recorded the response of live HTC cells to hypotonic exposure over time (in a room that was temperature-controlled to 19 °C). Figure 1A depicts three-dimensional volume projections of cells initially under isotonic conditions (300 mOsm) and then perfused with hypotonic solution (240 mOsm). Following hypotonic exposure, membrane protrusions transiently formed along the sides of the cells. (A video of this experiment is available in Supplemental Material). The protrusions expanded and contracted over a 3 min period (thin arrows), and subsequently could not be detected. Intriguingly, protrusions appeared to occur at different parts of the same cell, often adjacent to previous protrusions (such a protrusion is marked with a thick arrow). After 4 min, few membrane protrusions were visible. Quantitative analysis of cell volume revealed that the onset of visible membrane protrusions occurred at the start of cell swelling (Figure 1B). When protrusion formation ceased, cell volume recovery was already well underway, but not completed. Thus, HTC cells exposed to hypotonic conditions form transient membrane protrusions coincident with the increase in cell volume, but these cease before volume recovery is final.

Activated PLCγ associates with Src upon cell swelling — Since Src tyrosine kinases can be activated by integrins and subsequently can induce the activation of PLCγ, and PLCγ is activated by cell swelling (3), we sought to determine whether Src may play a role in volume-sensitive PLCγ activation. First we tested whether Src was activated upon cell swelling. Following hypotonic exposure, the amount of activated Src kinase in cell lysates, as determined by immunoblot analysis, rapidly peaked and fell to basal values (data not shown). To assess whether Src associated in a complex with PLCγ in response to hypotonic stimulus, we immunoprecipitated endogenous Src with a pp60Src antibody and then probed an immunoblot with activated Src and activated PLCγ antibodies. In response to hypotonic challenge, activated PLCγ co-immunoprecipitated with pp60Src. Both activated Src and activated PLCγ in the immunoprecipitate rapidly increased within 30 sec, and peak activation occurred at approximately 1-2 min (Figure 2A). (The timecourse of activation was more rapid than the appearance of membrane protrusions from the live cell work (done at 19 °C), but similar to immunofluorescence findings, which were also performed at room temperature, c.f. Figure 3). Of note, the kinetics of swelling-induced Src and PLCγ activation were similar. As shown in Figure 2B, there was a significant correlation between tyrosine phosphorylation of Src and of PLCγ in Src immunoprecipitates within the first 4 min following hypotonic exposure. To confirm that Src activation correlated with its membrane association, we examined the amount of activated Src from crude membrane fractions and from cytosolic fractions of lysates (Figure 2C). In response to hypotonic exposure, a time-dependent increase in activated Src was seen in membrane but not cytosolic fractions, with kinetics similar to those described above. Together, these observations suggest that cell swelling elicits transient increases in Src association with the membrane, by Src activation by tyrosine phosphorylation, and in Src association with PLCγ.

Swelling elicits Src activation in transient membrane protrusions — To determine whether the transient changes in Src activation and membrane association bore any relationship to the transient membrane protrusions, we performed a series of immunofluorescence experiments. First cells with and without hypotonic treatment were fixed and stained with the anti-pp60Src to determine whether endogenous Src could be found to be associated with the transient de novo protrusion structures on cell swelling. Immunofluorescence signal from total endogenous Src and actin showed the presence of Src puncta within membrane protrusions at the earliest time points after swelling (Figure 3A), similar to the activation time course observed by immunoblot analysis (Figure 2C). Thus, endogenous Src was present within the transient membrane protrusions at the earliest time points after hypotonic treatment.

Next we sought to determine whether Src was activated at the membrane of the swelling-induced membrane protrusions. Because the pattern of endogenous activated Src was difficult to see with immunofluorescence, cells were transiently transfected with c-Src to improve the signal to noise. Cells with the lowest detectable
levels of expression were chosen to avoid Src overexpression phenotypes. Following hypotonic exposure, elevated activated Src signal (using the rabbit anti-phosphotyrosine-Src antibody) was visible at the plasma membrane along the sides of transiently transfected cells, especially during the very earliest time points after hypotonic exposure (Figure 3B). Moreover, it was concentrated in obvious protrusive membrane structures (Figure 3B, small arrows). Intriguingly, these structures were particularly weakly labeled for actin, and appeared separated from the main cortical actin cytoskeletal layer typically located directly beneath the plasma membrane. (The results were consistent with experiments performed in which staining for endogenous activated Src with a monoclonal anti-phosphotyrosine-Src antibody was employed, cf. Figure 4). To confirm that the morphologies of cells with low levels of c-Src overexpression appeared similar to those of untransfected cells under isotonic conditions, we stained transfected cells without hypotonic treatment. Cells stained for activated Src showed an expected pattern of accumulation at the leading edge of lamellipodia at the base of migrating cells (Figure 3C). The shapes of these cells, as visualized by F-actin staining, were similar to the untransfected cells and not suggestive of a Src-transformed phenotype (i.e. rounded up and spindle-shaped). In contrast to the hypotonically treated c-Src transfected cells, little activated Src signal was seen above the base of the cell under isotonic conditions. Together, these findings suggested that cell swelling elicits the transient activation of Src at the plasma membrane above the base of the cell, in part, in transient membrane protrusions.

Activated Vav colocalizes with activated Src in membrane protrusions — In fibroblasts, the formation of membrane protrusions in swollen cells requires activation of the Rho GTPases Cdc42 and Rac1, and this process appears to involve engagement of integrins (4), similar to Cdc42 and Rac1-regulated protrusion formation at the leading edge of migrating cells (40). Vav is a RhoGEF that is activated by integrin engagement through Src (27). Since swelling can activate Src in an integrin-dependent manner (5), we tested whether activated Vav localized to membrane protrusions in response to cell swelling.

Figure 4 illustrates the effect of hypotonic exposure on the pattern of endogenous activated Vav immunofluorescence and its relation to that of endogenous activated Src. Under isotonic conditions (0 min), most cells had relatively low amounts of activated Src (using the monoclonal anti-phosphotyrosine-Src antibody) signal, especially above the base of the cell (Figure 4A). The exceptions were rare presumptive mitotic cells, which showed uniformly dispersed patterns of activated Vav and activated Src signal (data not shown), consistent with the cell cycle roles described for members of each these families of proteins (41-43). In response to hypotonic exposure, there was a transient increase in the intensity of activated Vav staining, and activated Vav colocalized with activated Src in membrane protrusions above the base of the cells (Figure 4A). The time-dependent increase in the colocalization of activated Vav and activated Src (Figure 4B) paralleled the swelling-induced increase in the proportion of cells with at least one membrane protrusion, which rose from 0 to 80% within 15 sec (data not shown).

At the base of the cells (Figure 4A), activated Vav and activated Src began to colocalize in small apparent focal complexes/adhesions at longer timepoints after hypotonic exposure, which by 4 min developed into larger apparent focal adhesion structures. By the time membrane protrusions had resolved, activated Vav and activated Src signal were substantially diminished on the sides of the cells above the base. Thus, during swelling, activated Vav first colocalized with activated Src in membrane protrusions above the base of the cells. At later times in cell volume recovery, activated Vav and activated Src were colocalized at the base of the cells, associated with apparent focal adhesion formation.

To determine whether the formation of apparent activated Vav-associated focal adhesions seen at 4 min was a general feature of cell volume recovery, we tested much later time points after hypotonic treatment. At 20 min (Figure 5A), large focal adhesion-like structures could be seen at the base of the cell, staining positively for both activated Vav and activated Src. At this time, thick filaments of bundled actin could be seen connecting some of these structures, consistent with formation of stress fibers (Figure 5A,
To determine whether these structures were classic focal adhesions, we stained cells under isotonic conditions for activated Vav, focal adhesion kinase (FAK), and F-actin. Shown in Figure 5B are images of the base of cells labeled with antibody directed against activated Vav (activated Vav signal was disperse above the base, data not shown). The pattern of activated Vav staining overlapped with that of FAK in the largest focal adhesion-like structures, and some of these were also connected with bundled F-actin fibers (Figure 5B, small arrows). In analogously prepared cells, the pattern of activated FAK more closely resembled that of activated Vav than of FAK, and activated FAK colocalized with activated Src, consistent with previous reports (44), (data not shown). These findings suggested that activated Vav is localized to focal complexes at the base of the cell under isotonic conditions and at late times after hypotonic exposure, when much of cell volume recovery has occurred. Activated Vav and activated Src were also found in additional subcellular localizations under isotonic conditions. Consistent with previously published roles for Vav and Src in cell migration, activated Vav and activated Src colocalized in lamellipodia and the earliest actin-based structures, as seen in a migrating cell depicted in Figure 5C. Taken together, these results suggest that during cell swelling, activated Vav first colocalized with activated Src in membrane protrusions above the base of the cells. At later times in cell volume recovery, activated Vav and activated Src were colocalized at the base of the cells associated with focal adhesions.

**Vav is a volume-sensitive effector for Src** — To determine whether Src and Vav were associated in a common complex, we performed immunoprecipitation of Src and subsequent immunoblot analysis for activated Vav and activated Src following hypotonic exposure. As shown in Figure 6A, activated Vav was detected in pp60Src immunoprecipitates of cell membrane fractions, and its abundance rapidly increased following hypotonic exposure. The kinetics of the activation of Src-associated Vav closely paralleled those of the activated Src. As shown in Figure 6B, there was a significant correlation between tyrosine phosphorylation of Vav and of Src in Src immunoprecipitates within the first 4 min following hypotonic exposure. By contrast, the abundance of activated Vav in cytosolic fractions was unaffected by hypotonic exposure (data not shown). Taken together, these observations suggested that the colocalization of activated Vav and Src in swelling-induced membrane fractions reflected the association and spatial relocalization of these proteins in a volume-sensitive signaling complex at the plasma membrane.

If Src kinase activity were responsible for swelling-induced Vav phosphorylation, it would be predicted that Src inhibition would prevent this process. To test this, we examined the effects of the Src kinase inhibitor PP2 on tyrosine phosphorylation of Vav in response to hypotonic exposure. In the presence of PP2 (10 µM), Vav tyrosine phosphorylation following hypotonic exposure was markedly attenuated (Figure 6C). By contrast, swelling-induced Vav tyrosine phosphorylation was not prevented by PP3 (10 µM), a PP2 analog that does not inhibit Src family kinases. These data suggested that activation of Vav in response to swelling is dependent on Src activity. By extension, these findings are consistent with a role for Src, via its effects on Vav, to control volume-sensitive actin remodeling.

**PLCγ is another volume-sensitive effector for Src** — We have previously shown in HTC cells that hypotonic challenge produces a transient increase in activated PLCγ, which in turn, triggers intracellular Ca2+ mobilization that is necessary for activation of volume-sensitive K+ and Cl– channels (3). Our findings above indicate that PLCγ is another volume-sensitive effector for Vav, to control volume-sensitive actin remodeling. PLCγ activation was Src-dependent, we tested the effect of Src inhibition on formation of activated PLCγ. As shown in Figure 7, PP2 (10 µM) prevented the swelling-induced time-dependent increase in the abundance of activated PLCγ. By contrast, PP3 (10 µM) did not prevent this increase. Collectively, these findings imply that Src is required for volume-sensitive activation of PLCγ. By extension, it would be predicted that Src, via its effects on PLCγ, controls the activation of volume-sensitive K+ and Cl– channels.

**Src activity is required for volume-sensitive ion channel activation and cell volume recovery** — In
order to determine whether Src was necessary for activation of volume-sensitive K⁺ and Cl⁻ channels, we examined the effects of Src inhibition on membrane currents following cell swelling. As shown in Figure 8A, PP2 (10 µM) inhibited swelling-activated K⁺ and Cl⁻ currents in HTC cells by over 80% (p<0.05, compared with control conditions). By contrast, PP3 (10 µM) did not significantly attenuate these currents. These findings thus support a requirement for Src in volume-sensitive ion channel activation and are consistent with a requirement for Src in volume recovery.

Finally, to assess whether cell volume recovery required Src activation, we tested the effects of Src inhibition on this process. PP2 (10 µM) significantly inhibited volume recovery in HTC cells, whereas PP3 (10 µM) did not (Figure 8B). Collectively, these findings indicate that Src is a key regulator of volume recovery following hepatocellular swelling.

DISCUSSION

Osmotic challenge evokes dynamic changes in the actin cytoskeleton and also activates fluid and electrolyte efflux to restore cell volume toward its resting state. This study tested whether Src might control these distinct cell volume regulatory pathways. We found that hypotonic exposure elicited a transient increase in activated Src, which first associated with the plasma membrane and in membrane protrusions along the sides of the cell, and later associated with focal adhesions at the base of the cell. Src activation exhibited initial kinetics similar to the activation of PLCγ and Vav, and these proteins colocalized with activated Src. The Src inhibitor PP2, but not its analog PP3, attenuated activation of Src, Vav, PLCγ, volume-sensitive K⁺ and Cl⁻ channels, and suppressed volume recovery. These observations suggest that cell swelling elicits activation of Src to coordinate at least two distinct pathways in volume recovery: cytoskeletal reorganization, via Vav, and volume-sensitive ion channel activation, via PLCγ.

The link between Src-mediated activation of Vav and volume-sensitive cytoskeletal reorganization has been previously seen in cell migration, where it drives membrane protrusion. In that step, Src activates Vav, which activates Rac1 at the leading edge of spreading cells (27). In addition to Rac1, Vav can activate other Rho GTPases, including Cdc42, and RhoA (29). Both Cdc42 and Rac1 are required for membrane protrusion formation in response to cell swelling (4), and since both can form different effector complexes that regulate the actin polymerization module at the front of spreading cells (45), it is likely that the cell protrusion targets of Src and Vav during cell swelling are Cdc42 and Rac1. Intriguingly, Cdc42 and Src may also interact to control subsequent membrane retraction, since activated Cdc42 can modulate both tyrosine phosphorylation and dephosphorylation of Src family kinases (46). Taken together, swelling-induced Src activation of Vav at the periphery of membrane protrusions, as we have observed, may be analogous to cell spreading-induced Src activation of Vav during lamellipodium formation.

Our observations also suggest that Src and Vav interact with RhoA at the base of the cell during cell volume recovery, as Src and Vav become associated with apparent focal adhesions. The latter are regulated by RhoA, in contrast to initial peripheral focal complexes, which are regulated by Cdc42 and Rac1 (47). The activated Vav structures that form at such a time (Figure 5A) are likely to be RhoA-regulated focal adhesions, because of their position, size and shape, they co-localize with FAK, and they are connected by thick filaments of bundled actin, consistent with stress fibers undergoing RhoA-ROCK medicated contractile forces (48). Thus, it is likely that at late times during volume recovery, activated Src and activated Vav act on RhoA at the base of the cell to provide contractile forces to further organize the cytoskeleton. If this is indeed the case, then in response to cell swelling, Src is activated at different parts of the cell at different times to control distinct Rho GTPases through the same activator, Vav.

Based on work by others (4,5), integrin activation is likely to be responsible for Src stimulation by cell swelling. If so, our data suggest that volume-sensitive integrin activation of Src leads to activation of Vav by tyrosine phosphorylation (cf. Figure 6). This contrasts with reports using extracellular matrix stimulation of integrins, in that no such increase in tyrosine
phosphorylation of Vav has been observed, even though integrin engagement leads to Src-dependent Vav activation (27,49). We found that the swelling-induced increase of Vav tyrosine phosphorylation was detectable only in membrane fractions. Since previous studies with matrix were performed on unfractionated samples, it is possible that analysis of membrane fractions might lead to detectable increases in Vav tyrosine phosphorylation under the conditions of Src activation through integrin engagement of extracellular matrix.

Our findings suggest a distinct volume-regulatory function for Src in the tyrosine phosphorylation of PLCγ, which we have previously shown to trigger intracellular Ca²⁺ mobilization that is necessary for activation of volume-sensitive K⁺ and Cl⁻ channels (3). How Src activates PLCγ remains an open question. It is intriguing that we have previously observed swelling-induced PLCγ membrane localization (3) in structures (early in protrusions, later in focal adhesions) similar to those observed for Src and Vav in the present study. Moreover, our current observations demonstrate that Src and PLCγ can be immunoprecipitated together in a signaling complex. Although these findings are consistent with the concept that Src directly regulates PLCγ activity following cell swelling, it is possible that another signaling molecule, which binds Src, in fact phosphorylates and activates PLCγ. Indeed, a recent study suggests the essential participation of a scaffolding protein, GIT1, in Src-dependent PLCγ activation (50). GIT1 is constitutively associated with PLCγ, and its phosphorylation by activated Src leads to a conformational change that either recruits another tyrosine kinase or that facilitates Src phosphorylation of PLCγ (50). Interestingly, not only does activated GIT1 function to regulate prosuractive activity and cell migration, but it is localized both to the leading edge of protrusions and to focal adhesions (51), analogous to PLCγ-associated structures that we have previously observed following cell swelling (3). Another possibility is that Src may regulate PLCγ through Vav, as has been described in T lymphocytes and mast cells (52,53). We believe this is less likely, since the adaptor molecules that mediate the association between Vav and PLCγ in cells of hematopoietic origin (i.e., LAT, SLP-76) do not appear to be expressed in non-hematopoietic tissues (54), whereas GIT1 is ubiquitously expressed. Finally, it is conceivable that the Src inhibitor PP2 prevents PLCγ phosphorylation via a mechanism unrelated to its effects on Src activity. However, the most parsimonious explanation for our collective findings is that volume-sensitive Src activation mediates tyrosine phosphorylation of PLCγ.

It is highly likely that Src regulates volume recovery through mechanisms in addition to that of activation of Vav and PLCγ. Intriguingly many of these effectors have also been implicated in cell migration processes. The extension and contraction of the membrane protrusions may be controlled by Src phosphorylation of the WASP/WAVE effectors of Cdc42 and Rac1 (55,56). FAK, a Src substrate, has been reported to undergo tyrosine phosphorylation upon hepatocellular swelling (57,58), and may inhibit RhoA through p190RhoGAP during the initial phases of volume recovery (14,59). Another Src substrate, phosphatidylinositol (PI) 3-kinase, is rapidly activated upon hepatocellular swelling and triggers volume-sensitive ATP release, which in turn elicits the opening of volume sensitive Cl⁻ channels via purinergic signaling pathways (60). Moreover, PI 3-kinase is also implicated in the regulation of many cytoskeletal modulators, and is localized to the leading edge of migrating cells (61). An additional mechanism by which Src may coordinate volume-sensitive fluid and electrolyte efflux with cytoskeletal rearrangement is through direct Src-mediated phosphorylation of volume-sensitive ion channels, as it does in the activation of neuronal large conductance Ca²⁺-activated K⁺ channels (62).

By integrating our findings with observations by others, we propose the following model (Figure 9). Cell swelling leads to the activation of Src and its recruitment with other signaling molecules to the periphery of the cell. Activated plasma membrane-associated Src can then coordinate reorganization of the actin cytoskeleton, through Vav-mediated activation of Cdc42 and Rac1, as well as trigger fluid and electrolyte efflux through PLCγ-mediated activation of volume-sensitive K⁺ and Cl⁻ channels. As volume recovery begins, Src is then
activated at the base of the cell to stimulate Vav to reform mature focal adhesions with RhoA.

Just as events in cell migration require temporally and spatially-controlled activation of cytoskeletal effectors with other processes, so must volume recovery involve an integrated response of reorganization of the cytoskeleton with restoration of fluid homeostasis. We believe that Src functions as the trigger and as a master controller in cell volume recovery, adapting diverse signaling modules to achieve this end.
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FOOTNOTES

1The abbreviations used are: PLCγ, phospholipase Cγ; GEF, guanine nucleotide exchange factor; IP3, inositol 1,4,5-trisphosphate; AM, acetoxymethyl ester; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine; SES, standard extracellular solution; HES, hypotonic extracellular solution; %RVD, percent volume recovery; FAK, focal adhesion kinase; PI, phosphatidylinositol; pY-PLCγ, activated PLCγ; pY-Src, activated Src; IP, immunoprecipitation; pY-Vav, activated Vav.

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FIG 1. Hypotonic challenge elicits transient formation of plasma membrane protrusions. A. HTC cells were loaded with calcein and imaged at 19°C in real time by fluorescence microscopy. Twenty 1 µm z-sections were collected every minute, deconvolved, and volume projections were reconstructed from the data. Shown are timepoints from one movie of cells exposed to hypotonic solution, which was administered at 0 min (see B below). Thin arrows mark two different plasma membrane protrusions that expand and contract over 3 min. The thick arrow marks a protrusion that developed adjacent to a previous protrusion. The scale bar represents 20 µm. B. The volume of each of the four cells above was calculated under isotonic conditions (prior to time = 0) and following hypotonic exposure (bar). Note that the onset of membrane protrusions in A correlated with the onset of swelling, and that membrane protrusions ceased before volume recovery was complete.

FIG 2. Src undergoes rapid tyrosine phosphorylation and membrane association following hypotonic exposure. A. A representative immunoblot of pp60-Src immunoprecipitates of lysates from cells exposed to hypotonic solution for different times, and probed for activated PLCγ (pY-PLCγ), activated Src (pY-Src), and total Src. B. The correlation between pY-PLCγ and pY-Src in Src immunoprecipitates is significant during the first 4 min after hypotonic exposure (r = 0.700, p = 0.004 by Spearman nonparametric analysis). Band intensities of individual time points from 3 experiments performed as in A were obtained by densitometry, and the pY-PLCγ and pY-Src signals were each divided by the fold increase in pp60-Src signal (relative to basal conditions). Data are expressed as relative densitometric units (× 10^4). C. Shown are representative immunoblots of crude cytosolic and membrane fractions from cells exposed to hypotonic solution for different times. The blots were probed for pY-Src, pp60-Src and actin.

FIG 3. Localization of Src in response to hypotonic exposure. A. Endogenous Src is found in membrane protrusions upon swelling. Cells grown on coverslips were treated with hypotonic media for different times at room temperature, fixed, and stained for endogenous Src (red, CY3) and for F-actin (green, FITC-phalloidin). Twenty z steps of 1 µm were taken and deconvolved. The images shown are of the 1 µm section that is 4 µm above the base of the cell. The 0.25 min and 0.5 min split panels indicate that boxed cells were moved from the same field to fit the depiction at this magnification: 30 µm to the left and 7 µm up respectively. The scale bar is 15 µm. B. Activated Src is found in swelling-induced protrusions on the sides of cells. c-Src transfected cells were exposed to hypotonic solution for different amounts of time prior to fixation and staining for activated Src (pY-Src, red, CY3), using the rabbit anti-phosphotyrosine-Src antibody, and F-actin (green, FITC-phalloidin). Protrusions with elevated activated Src signal are marked with arrows. The images shown correspond to 0.2 µm z sections 3.6 to 4 µm above the base of the cells. The scale bar is 15 µm. C. Activated Src under isotonic conditions is predominantly at the base of the cell, as opposed to the sides of the cell. Cells were transiently transfected with c-Src, and treated as in A, except that an antibody against activated Src (using the rabbit anti-phosphotyrosine-Src antibody) was used in place of the antibody against total Src. Shown are 0.2 µm z sections of cells stained for pY-Src (red, CY3) and F-actin (green, FITC-phalloidin) at the base and 4 µm above the base. The scale bar is 15 µm.
FIG 4. **Localization of Vav in response to hypotonic exposure.** A. Hypotonic exposure transiently increases endogenous activated Vav and endogenous activated Src in different locations within cells at different times. Cells grown on coverslips were treated with hypotonic media, fixed for immunofluorescence, and stained for activated Vav (pY-Vav, red, CY3) and activated Src (pY-Src, green, Alexa 488), using the monoclonal anti-phosphotyrosine-Src antibody. Images of 0.5 μm sections of cells fixed at different time points following hypotonic exposure are shown, taken from the base and 2 to 6.5 μm above the base. The scale bar represents 10 μm. In the images depicted, gamma adjustment of brightness and contrast was performed to improve the clarity of the visualization despite low signal. B. Quantification of pY-Src and pY-Vav colocalization. Cells were treated with hypotonic media and fixed and stained as above. The images were deconvolved, three dimensional projections were generated, and the overlapping voxels occupied by pY-Src and pY-Vav signals were quantified. Data represent the means ± SE of 3 fields of cells (6-9 in each field). The asterisks connote statistical significance, compared with basal values (p < 0.05).

FIG 5. **Localization of Vav in focal adhesions** A. Vav is relocalized to apparent focal adhesions during cell volume recovery. Cells fixed 20 min after hypotonic exposure reveal that activated Src (pY-Src, green, CY3), using the monoclonal anti-phosphotyrosine-Src antibody, and activated Vav (pY-Vav, blue, Alexa 488) are found in large focal adhesion-like structures connected to actin filaments (red, Alexa 687-phalloidin). Images are of a 0.5 μm section of cells at the base. The thin arrows mark a thick actin filament between two such structures. The scale bar represents 15 μm. B. Under isotonic conditions, activated Vav is found in structures that also contain FAK. Small arrows indicate two focal adhesions with actin fibers between them. Cells grown on coverslips were fixed and stained for pY-Vav (red, CY3), as above, FAK (FAK, green, Alexa 488), using an anti-FAK antibody, and F-actin (blue, Alexa 647-phalloidin). Depicted are images of 0.5 μm sections at the base of cells. The scale bar represents 10 μm. C. In migrating cells under isotonic conditions, activated Vav and activated Src are found associated with actin structures at the periphery of cells. Cells grown on coverslips under isotonic conditions were fixed and stained for pY-Vav (green, Alexa 488), pY-Src (red, CY3), using the monoclonal anti-phosphotyrosine-Src antibody, and F-actin (blue, Alexa 647-phalloidin). The image represents a 0.5 μm section at the base of a cell. The tip of an F-actin-based structure is marked with an arrow. The scale bar represents 10 μm.

FIG 6. **Src mediates volume-sensitive Vav activation.** A. Src and Vav are associated in a common complex upon cell swelling. After exposure to hypotonic solution for different timepoints, cells were fractionated into cytosolic and membrane fractions, and immunoprecipitation was performed using pp60Src antibody. Shown is a representative immunoblot from a membrane-fraction immunoprecipitation, probed with antibodies against activated Vav (pY-Vav) and activated Src (pY-Src). B. The correlation between pY-Vav and pY-Src in Src immunoprecipitates is significant during the first 4 min following hypotonic exposure (r = 0.798, p < 0.001 by Spearman nonparametric analysis). Band intensities of individual time points from 3 experiments performed as in A were obtained by densitometry, and the pY-Vav and pY-Src signals were each divided by the fold increase in pp60-Src signal (relative to basal conditions). Data are expressed as relative densitometric units (× 10^3). C. Activation of Vav requires Src. Cells were exposed to hypotonic solution in the presence and absence of the Src kinase inhibitor, PP2 (10 μM), or its analog, PP3 (10 μM), and harvested at different timepoints and fractionated. The cytosolic and membrane fractions were run on gels, transferred to membranes, and probed for pY-Vav, pY-Src, and pp60-Src.

FIG 7. **PLCγ activation upon swelling is Src-dependent.** Cells were exposed to hypotonic solution in the presence and absence of PP2 (10 μM) or PP3 (10 μM), and cell lysates prepared at different timepoints. A representative immunoblot of a phosphotyrosine immunoprecipitation was probed for activated PLCγ (pY-PLCγ, activated Src (pY-Src), and mouse immunoglobulin (mlg).
FIG 8. **Inhibition of Src attenuates swelling-induced membrane K⁺ and Cl⁻ currents and cell volume recovery.** A. K⁺ and Cl⁻ currents were measured by patch clamp recording techniques and normalized to cell capacitance (see Experimental Procedures). Currents were obtained under basal isotonic conditions and 10 min after the onset of hypotonic exposure in the presence or absence of PP2 (10 µM) or PP3 (10 µM). Data represent the means ± SE of 6-8 cells for each condition. The asterisks connote statistical significance, compared with basal values (p < 0.05), and NS indicates not statistically significant. B. Src inhibition attenuates volume recovery after hepatocellular swelling. Relative cell area was determined by fluorescence image analysis in calcein-loaded cells, under isotonic conditions and during hypotonic exposure (hatched bar) in the absence (control) or presence of PP2 (10 µM) or PP3 (10 µM). Recovery from swelling (%RVD, as defined in Experimental Procedures) was significantly less with PP2 (16.8 ± 1.6, n = 38 cells) than under control conditions (33.1 ± 2.7, n = 39 cells) or with PP3 (29.1 ± 3.2, n = 44 cells).

FIG 9. **Model for volume-sensitive Src-mediated signaling.** Cell swelling elicits Src activation, which in turn activates Vav and PLCγ. Vav mediates cytoskeletal reorganization as a consequence of its activation of Rho GTPases at different times and places within the cell, while PLCγ mediates the activation of volume-sensitive K⁺ and Cl⁻ channels and osmotically-driven fluid efflux. Together, these effectors regulate cell volume recovery.

**SUPPLEMENTAL FIGURE LEGENDS**

**SUPPLEMENTAL FIG 1. Properties of c-Src transfected HTC cells.** The upper panels show c-Src transfected cells (red, activated Src) in a field of untransfected cells (all cells stain for actin, green) under isotonic and hypotonic conditions. Only cells that stain red are transfected with c-Src; untransfected cells do not show staining with this antibody (the rabbit activated Src antibody). The lower panels show HTC cell morphologies resulting from different levels of c-Src expression. The panels on the left show a c-Src transfected cell (red) that appears to be migrating to the left. This cell exhibits strong activated Src signal at the lamellipodium at the front end of the cell, where activated Src has a well-documented function. In the panel on the right are two c-Src transfected cells (red). One cell is well-adhered, but the other stains brightly for activated Src and is largely above the plane of the rest if the cells. This poorly adhered, spindly cell would not be chosen for analysis, because it has the aberrant morphology characteristic of Src-transformed cells.

**SUPPLEMENTAL FIG 2. Protrusion frequency in swollen HTC cells.** The bar graph shows the number of cells scored with at least one protrusion 1 µm or more above the base of the cell. Panels on the right show three-dimensional projections of different fields of cells stained for endogenous activated Src (green) or activated Vav (red). The top panel with isotonically treated cells show relatively little colocalization (yellow), in contrast to the bottom four panels which show different times of hypotonically-treated cells.

**SUPPLEMENTAL DATA FIGURE LEGEND**

The video (video1.mov) represents a time-lapse image of the uppermost cell in depicted in Fig.1. and demonstrates reversible membrane protrusions that form upon hypotonic challenge.
Figure 1

A

0 min 1 min 2 min 3 min
4 min 5 min 6 min 15 min

B

Hypotonic

Cell Volume (picoliters)

Time (min)

3000 6000
Figure 3

A

0 min 0.25 min 0.5 min
1 min 2 min 4 min

B

0 min 0.25 min 0.5 min 1 min 2 min 4 min

pY-Src

F-actin

Merge

C

Base

Above Base
Figure 4

A

| Time  | Base | Above Base |
|-------|------|------------|
|       | Merge | Merge      | pY-Src | pY-Vav |
| 0 min | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| 0.25 min | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| 0.5 min | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| 1 min  | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |
| 2 min  | ![Image](image17) | ![Image](image18) | ![Image](image19) | ![Image](image20) |
| 4 min  | ![Image](image21) | ![Image](image22) | ![Image](image23) | ![Image](image24) |

B

![Graph showing overlap over time](image25)
Figure 5

A  
20 min Hypotonic

B

C
**Figure 6**

(A) Western blot analysis showing changes in phosphorylation of Vav and Src over time (0 to 8 minutes) with IP: pp60Src.

(B) Graph depicting the relationship between pY-Vav and pY-Src with time.

(C) Western blot analysis of Vav, Src, and pY-Vav under control and treated conditions (PP2 and PP3) at different time points (0, 0.5, 2 minutes) in Cytosol and Membrane fractions.
Figure 7

| Time (min) | Control | PP2 | PP3 |
|-----------|---------|-----|-----|
| 0 | ![Control pY-PLCγ](image) | ![PP2 pY-PLCγ](image) | ![PP3 pY-PLCγ](image) |
| 2 | ![Control pY-PLCγ](image) | ![PP2 pY-PLCγ](image) | ![PP3 pY-PLCγ](image) |
| 4 | ![Control pY-PLCγ](image) | ![PP2 pY-PLCγ](image) | ![PP3 pY-PLCγ](image) |
| 0 | ![Control pY-Src](image) | ![PP2 pY-Src](image) | ![PP3 pY-Src](image) |
| 2 | ![Control pY-Src](image) | ![PP2 pY-Src](image) | ![PP3 pY-Src](image) |
| 4 | ![Control pY-Src](image) | ![PP2 pY-Src](image) | ![PP3 pY-Src](image) |
| 0 | ![Control mlg](image) | ![PP2 mlg](image) | ![PP3 mlg](image) |
| 2 | ![Control mlg](image) | ![PP2 mlg](image) | ![PP3 mlg](image) |
| 4 | ![Control mlg](image) | ![PP2 mlg](image) | ![PP3 mlg](image) |

IP: phosphotyrosine
c-Src transfected HTC cells

red: activated Src
green: actin

isotonic

hypotonic

activated Src

merge
Src regulates distinct pathways for cell volume control through Vav and phospholipase C gamma

Elisabeth T. Barfod, Ann L. Moore, Richard F. Melnick and Steven D. Lidofsky

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