Cell Shrinkage Regulates Src Kinases and Induces Tyrosine Phosphorylation of Cortactin, Independent of the Osmotic Regulation of Na\(^+/\)H\(^+\) Exchangers

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The signaling pathways by which cell volume regulates ion transporters, e.g. Na\(^+/\)H\(^+\) exchangers (NHEs), and affects cytoskeletal organization are poorly understood. We have previously shown that shrinkage induces tyrosine phosphorylation in CHO cells, predominantly in an 85-kDa band. To identify volume-sensitive kinases and their substrates, we investigated the effect of hypertonicity on members of the Src kinase family. Hypersmolality stimulated Fyn and inhibited Src. Fyn activation was also observed in nystatin-permeabilized cells, where shrinkage cannot induce intracellular alkalinization. In contrast, osmotic inhibition of Src was prevented by permeabilization or by inhibiting NHE-1. PP1, a selective Src family inhibitor, strongly reduced the hypertonicity-induced tyrosine phosphorylation. We identified one of the major targets of the osmotic stress-elicted phosphorylation as cortactin, an 85-kDa actin-binding protein and well known Src family substrate. Cortactin phosphorylation was triggered by shrinkage and not by changes in osmolarity or pH, and was abrogated by PP1. Hypersmotic cortactin phosphorylation was reduced in Fyn-deficient fibroblasts but remained intact in Src-deficient fibroblasts. To address the potential role of the Src family in the osmotic regulation of NHEs, we used PP1. The drug affected neither the hyperosmotic stimulation of NHE-1 nor the inhibition of NHE-3. Thus, members of the Src family are volume-sensitive enzymes that may participate in the shrinkage-related reorganization of the cytoskeleton but are probably not responsible for the osmotic regulation of NHE.

The maintenance of normal cell volume is an essential homeostatic function. Most cells are equipped with a variety of volume-sensitive membrane transporters (e.g. isoforms of the Na\(^+/\)H\(^+\) exchanger, Na\(^+\)/K\(^+\)/Cl\(^-\) cotransporter, K\(^+\) and Cl\(^-\) channels) that can effectively restore normal cell size after a perturbation caused either by exposure to an aniso-osmotic environment or by metabolic changes (for reviews, see Refs. 1 and 2). Alterations in the cell volume are also known to induce reorganization in the actin skeleton (3–6) and to modulate gene transcription (7–9). Moreover, the cellular hydration state is not only a subtly regulated parameter but is also an important regulatory signal (1). For example, hormones such as glucagon and insulin elicit volume changes which act as “second messengers” necessary for their cellular effects.

Little is known about the volume-dependent signaling mechanisms and their relationship to the different effector systems such as the ion carriers or the cytoskeleton. Evidence has been accumulating that protein-tyrosine kinases may play a pivotal role in the signaling of both hypo- (5, 10, 11) and hyperosmotic shock (12–14). Our recent studies using CHO cells (12) as well as that of Krump et al. on neutrophils (13) have shown that hypertonicity induces robust tyrosine phosphorylation in various protein bands. While the phosphorylation pattern differed in the two cell types studied, the trigger for the phosphorylation of most proteins was cell shrinkage and not an increase in osmolarity or in intracellular ion concentrations. In CHO cells, hypertonicity induced phosphorylation of proteins of -40, 85, and 110–130 kDa, with the most prominent response occurring in the ~85-kDa band (p85). While the ~40-kDa protein proved to be extracellular signal-regulated kinase-2, the identity of p85 and the other higher molecular weight proteins remains to be elucidated. Further, the kinase pathways responsible for these reactions are unknown. Some of the toxicity-sensitive proteins complexed with Src homology 2 (SH2) and SH3 domains, raising the possibility that the Src family of tyrosine kinases might be potential mediators of the osmotically induced phosphorylations. This notion is further strengthened by the finding that, in neutrophils, hypertonicity altered the activity of Fgr, Hck, and Lyn (13), members of the Src family expressed specifically in cells of hematopoietic origin. Furthermore, pharmacological data fostered the concept that the hypertonicity-stimulated tyrosine phosphorylation might be causally connected to the osmotic regulation of Na\(^+/\)H\(^+\) exchange. Specifically, the effect of hypertonicity on two osmotically sensitive, but oppositely regulated, isoforms of the Na\(^+/\)H\(^+\) exchanger (15–19) was abrogated by broad spectrum tyrosine kinase inhibitors; the hyperosmotic activation of Na\(^+/\)H\(^+\) exchanger-1 (NHE-1) (in neutrophils) was inhibited by genistein (13), whereas the hyperosmotic inhibition of NHE-3 (in kidney cells) was prevented by genistein and herbimycin (20). Based on these observations, Krump et al. (13) suggested that the hypertonic activation of NHE-1 in neutrophils may be due to...
the stimulation of certain Src kinases. To date, no information has been available about the osmotic responsiveness of the two most widely expressed, ubiquitous Src family members, p60 and p59. In addition, none of the major osmosensitive phosphoproteins has been identified. The aim of this study was to gain further insight into the mechanisms of volume-dependent signaling by investigating the potential role of the Src family in the hypertonicity-induced tyrosine phosphorylation and the subsequent changes in ion transport. Specifically, we intended to investigate whether Src and Fyn can be regulated by a decrease in cell volume and to ascertain whether the Src family may play an essential role in the osmotic regulation of NHE-1 or NHE-3. Our results show that members of the Src family are regulated by cell shrinkage, and one of the major targets for volume-dependent tyrosine phosphorylation is the Src family substrate, actin cross-linking protein, cortactin. On the other hand, the osmotic regulation of NHE-1 and -3 does not appear to be mediated by Src-like kinases.

EXPERIMENTAL PROCEDURES

Materials—MeSO2, nystatin (used from a stock solution of 400,000 units/ml in MeSO2, freshly prepared before each experiment), nigericin, monensin, N-methyl-D-glucammonium, and glucosonic acid lactone were purchased from Sigma. Proteinase inhibitor mixture containing 0.8 mg/ml benzamidine HCl, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 0.5 mg/ml phosphatase A, and 50 mM phenylmethylsulfonyl fluoride in pure ethanol was from PharMingen, Protein G-Sepharose beads from Amer sham Pharmacia Biotech, 2,7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)/acetoxyxymethylene and PPI were from Calbiochem. Monoclonal anti-phosphotyrosine (4G10), anti-cortactin, anti-p60, anti-p59, the Src assay kit including an Src family-specific substrate peptide, and the radiolime precipitation buffer were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-Fyn was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-phospho-p38 was from New England Biolabs. Peroxidase-conjugated anti-mouse and anti-rabbit IgG, the Enhanced Chemiluminescence kit, and [γ-32P]ATP (3000 Ci/mmol) were from Amersham Pharmacia Biotech.

Media—Bicarbonate-free RPMI 1640 was buffered with 25 mM Hepes (pH 7.4) or with 25 mM sodium bicarbonate (pH 7.4) at 37 °C. The Iso-Na medium consisted of 140 mM NaCl, 3 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 5 mM glucose, 20 mM Hepes (pH 7.4). When required, Iso-Na was made hypertonic (Hyper-Na, 600 mosm) by the addition of 300 mM sucrose. The Iso- and Hyper-K media had the same composition, except NaCl was replaced by KCl. The permeabilization medium contained 140 mM KCl, 1 mM MgCl2, 1 mM EGTA, 0.193 mM CaCl2 (100 mM free Ca2+), 5 mM glucose, 10 mM Hepes (pH 7.2). To permeabilize the cells, this medium was supplemented with 400 units/ml nystatin and 60 mM sucrose. Sucrose was included to counterbalance the intracellular colloidosmotic pressure and thereby prevent swelling of the permeabilized cells, as reported earlier by us (12). To induce shrinkage, the nystatin-containing permeabilization buffer was supplemented with 350 mM sucrose. The Iso-NMg medium was composed of 140 mM NMG, 140 mM glucoheonic acid lacton, 3 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 0.193 mM CaCl2 (100 mM free Ca2+), 5 mM glucose, 10 mM Hepes (pH 7.2). The osmolality of the isotonic solutions was adjusted to 290 ± 5 mosmol with the major salt. Osmolality was checked with an Osmette osmometer.

Cell Culture—For most studies, we used a CHO cell line (AP-1) derived of the endogenous NHE and stably transfected with the rat NHE-1 (referred to as NHE-1 cells) or NHE-3 (referred to as NHE-3 cells), as described previously (15). These cells were grown in a-α-methyl essential medium, containing 25 mM NaHCO3 and supplemented with 10% fetal calf serum, and 1% antibiotic suspension (penicillin and streptomycin; Sigma) under a humidified atmosphere of air/CO2 (19:1) at 37 °C. To eliminate potential revertants and to maintain the high expression level of the NHE isoforms, cells were selected after every passage for the Na+/H+ exchange-dependent survival of an acute acid load (15).

WT, Fyn−/−, Src−/− fibroblasts were isolated from mouse embryos that were homozygous for disruption in Src or Fyn gene and were immortalized with large T antigen (21). Cells were kindly provided by Sheila M. Thomas (Fred Hutchinson Cancer Center, Seattle, WA) and were maintained in Dulbecco’s modified Eagle’s medium. All other conditions and treatments were similar to those in CHO cells.

Human neutrophils were prepared from healthy volunteers as described previously (22) except that lysis of red blood cells was carried out using NH4Cl. Prior to use, cells (107/ml) were kept in Heps-buffered RPMI medium at 37 °C.

Preparation of Cell Extracts—Confluent cultures were incubated for at least 3 h in serum- and HCO3−-free RPMI 1640 prior to experiments. Cells were preincubated in Iso-Na medium for 10 min and then subjected to various treatments as indicated. The medium was then aspirated, and the cells were vigorously scraped into ice-cold Triton containing or non-Triton precipitation buffers, supplemented with 1 mM Na3VO4 and 20 μM protease inhibitor mixture. The Triton lysis buffer contained 100 mM NaCl, 30 mM Hepes, 20 mM NaF, 1 mM EGTA, 1% Triton X-100, pH 7.5, and the radiolime precipitation buffer was composed of 150 mM NaCl, 50 mM Tris-HCl, 1 mM NaF, 2 mM Tris-EGTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, pH 7.4.

Western Blotting and Immunoprecipitation—Lysates containing equivalent amount of protein were either mixed with an equal amount of 2× Laemmli buffer (whole cell lysates) or were clarified by centrifugation at 12,000 × g for 10 min and further processed for immunoprecipitation. Extracts were preclarihed for 1 h using 40 μl of 50% suspension of Protein G-Sepharose beads and then incubated with the corresponding antibodies (see details in the figure legends to Figs. 2–6) for 1 h. Immunocomplexes were captured using 40 μl of Protein G-Sepharose, washed the beads were washed with lysis buffer. Immunoprecipitated proteins were diluted with Laemmli sample buffer, boiled for 5 min, and subjected to electrophoresis on 10% SDS-polyacrylamide gels. The separated proteins were transferred to nitrocellulose using a Bio-Rad Mini Protein II apparatus. To check the effectiveness of transfer and similarity of protein amount, lanes were visualized by staining with Ponceau S. Blots were blocked in Tris-buffered saline containing 5% bovine serum albumin and then incubated with the primary antibody. The binding of the antibody was visualized by peroxidase-coupled secondary anti-mouse or rabbit antibody (1:3000 dilution) using the enhanced chemiluminescence method.

Densitometry—Quantification of the bands was performed using a Bio-Rad GS-690 imaging densitometer, and evaluation of data was carried out with the Molecular Analyser computer program (12).

In Vitro Kinase Assays—The activity of Src and Fyn was determined by immunocomplex kinase assays (23, 24). Cell lysates obtained from iso- or hypertonicity treated cells and containing equivalent amounts of protein (250–500 μg) were subjected to immunoprecipitation (see above), and the precipitates were washed with kinase buffer (20 mM HEPES, 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, pH 7.1). Kinase activity was measured as the phosphorylation of either the Src family-specific substrate peptide Cdc2-6(–20) or enolase. In the former case, the Upstate Biotechnology Src kinase assay kit was used according to the manufacturer’s instructions. Briefly, the immunocomplexes were incubated with 20 μl of reaction buffer (100 mM Tris-HCl, pH 7.2, 125 mM MgCl2, 25 mM MnCl2, 2 mM EGTA, 0.25 mM Na3VO4, 2 mM dithiothreitol, 0.1 μg of phosphotyrosine substrate peptide (0.6 mM stock in H2O), and the reaction was initiated by the addition of 10 μl of manganese/ATP mixture (0.5 mM ATP, 75 mM MnCl2 in reaction buffer) containing 10 μCi [γ-32P]ATP/sample. After 10 min at 30 °C, 20 μl of trichloroacetic acid was added, and 25 μl of the mixture was layered on P81 phosphocellulose squares. After extensive washing with 0.85% phosphoric acid, radioactivity bound to the filters was determined by scintillation counting. Nonspecific binding of radiolime to the filters was determined in each experiment by measuring the activity of samples to which 10 μl of H2O was added instead of the peptide. The low activity measured in these samples was subtracted as background. Experiments were repeated at least three times, and the results were normalized to the amount of protein content of the initial cell lysate. When enolase was used, the immunocomplexes were incubated with 8 μl of kinase buffer, 12 μl of acid-denatured enolase (1.75 μg of enolase/sample), and 10 μl of ATP mixture (kinase buffer supplemented with 3 μM K-ATP and 10 μCi of [γ-32P]ATP/sample). After 5 or 10 min at 30 °C for Src and Fyn, respectively, the reaction was terminated by the addition of 10 μl of 4× Laemmli buffer, and the samples were boiled and subjected to SDS-polyacrylamide gel electrophoresis. The gels were dried and used for direct quantification of radioactivity with the Molecular Dynamics Storm Imaging software. The gels were also subjected to radiography with an intensifying screen. Each experiment was performed at least three times, and all time duplicates or triplicates were measured. Results are expressed as fold increase compared with the controls.

Measurement and Manipulation of Cytoplasmic pH—pH was measured fluorometrically using the indicator dye BCECF, essentially as de
lysates were obtained and probed with a phosphospecific anti-p38 antibody. Incubated in Iso- or Hyper-Na medium, supplemented with 20 mM loading, in the presence or absence of PP1, cells were washed and then first acidified by the ammonium prepulse technique. After 10 min of dye incubation period, cells were lysed with ice-cold Triton lysis buffer, and the protein content of the samples was determined. After mixing with an equal amount of 2× Laemmli buffer, the various samples containing equal amounts of protein were subjected to electrophoresis, transferred to nitrocellulose, and probed with a monoclonal anti-phosphotyrosine antibody using enhanced chemiluminescence. The intensity of the 85-kDa band was quantified by densitometry and expressed as a fold increase compared with the isotonic sample. Data are means ± S.E. of three separate experiments. C, hyperosmolarity-induced tyrosine phosphorylation of p38 is not inhibited by PP1. Cells were treated as in A with the indicated concentrations of PP1. Whole cell lysates were obtained and probed with a phosphospecific anti-p38 antibody.

**Cell Shrinkage Regulates Src Kinases**

**RESULTS**

**The Effect of the Src Kinase Inhibitor, PP1, on Hypertonicity-induced Tyrosine Phosphorylation**—To assess whether Src kinases might play a role in the hypertonicity-induced tyrosine phosphorylation observed in CHO cells, we used PP1, a newly developed, selective pyrazolo pyrimidine-type inhibitor of this enzyme family (25, 26). Fig. 1A shows that osmotic shock evoked strong tyrosine phosphorylation of several proteins, the predominant response occurring in 80–85- and 110–130-kDa bands, as reported earlier by us (12). PP1 caused a concentration-dependent inhibition of the hypertonicity-triggered tyrosine phosphorylation in most bands. Half-maximal inhibition of p85 phosphorylation was obtained at ~2 μM, whereas 10 μM completely abolished phosphotyrosine accumulation in this band (Fig. 1B). This concentration dependence corresponds to the reported in vivo PP1 sensitivity of Src kinases (25, 26). The drug strongly reduced tyrosine phosphorylation in the 110–130-kDa region as well, but a part of the response was persistent even at concentrations as high as 100 μM. By contrast, PP1 (up to 100 μM) failed to affect the hypertonicity-elicited tyrosine phosphorylation of p38 stress kinase (Fig. 1C). These findings indicate that the toxicity-related tyrosine phosphorylations are carried out by at least two pharmacologically distinguishable signaling pathways, one of which is PP1-inhibitable, and thus potentially Src family-dependent, whereas the other is mediated by Src family-independent mechanisms.

**Cell Shrinkage Induces the Tyrosine Phosphorylation of the Src Family Substrate, Cortactin**—Given the PP1 sensitivity of p85 phosphorylation, we asked whether this band could be identified as a known Src family substrate. Cortactin, an 80/85-kDa actin cross-linking protein (27), is a preferred Src substrate, which has been reported to become intensively tyrosine-phosphorylated in fibroblasts overexpressing the oncogenic v-Src (28) or the mutationally activated Fyn (29) as well as in cells lacking Csk, a kinase that inactivates the Src family (30). Cortactin phosphorylation has also been observed in normal cells exposed to various stimuli known to activate c-Src or Fyn (31–35).

To address whether hypertonicity induces cortactin phospho-
by the addition of nystatin, an ionophore that selectively permeabilizes the membrane for small monovalent ions but does not allow the permeation of large organic ions. Fig. 2C demonstrates that isotonic shrinkage led to the marked tyrosine phosphorylation of cortactin. Together, these findings indicate that cell shrinkage is sufficient to elicit cortactin phosphorylation, independent of hypertonicity or the type of osmolytes used to achieve it.

To assess what portion of the 85-kDa osmosensitive band can be identified as cortactin, we immunodepleted hypertonic cell lysates with the anti-cortactin antibody and compared the level of reduction in the amount of cortactin and in the tyrosine-phosphorylated p85 band. The addition of 3-μg antibody and protein G-Sepharose beads to the Triton-soluble cell lysate (400 μg of protein) resulted in a 59 ± 3% decrease in the cortactin content of the sample (n = 3). Probing of the same blots with antiphosphotyrosine revealed that the anti-cortactin antibody caused a 34 ± 9% reduction in the p85 band as well, while it did not affect the intensity of other bands (not shown). Thus, more than half of the p85 band is composed of phosphocortactin. These studies therefore show that the Src family substrate cortactin is one of the major proteins undergoing tyrosine phosphorylation following osmotic shrinkage of CHO cells.

**The Effect of Hypertonicity on the in Vitro Activity of p60src and p59fyn**—Both our pharmacological data and the identification of cortactin as a phosphorylated substrate supported the notion that the Src family is involved in the mediation of the osmotically induced tyrosine phosphorylation. In further experiments, we investigated whether hypertonic treatment could in fact cause detectable changes in the activity of ubiquitously expressed Src kinases. Covalent modification (i.e. phosphorylation and dephosphorylation at inhibitory or activating tyrosine residues) of these kinases represents an important mechanism by which their activity is regulated (37). Changes in activity brought about by this type of control can be detected in vitro. To discern whether hyperosmotic shock may affect members of the Src family in this manner, we performed *in vitro* kinase assays on Src and Fyn immunoprecipitates obtained from iso- and hypertonicity-treated cells. Fig. 3 shows that hypertonic exposure of the cells (10 min, 600 mosM) caused a significant decrease (50%) in p60src activity. This response was not dependent on the Src substrate applied, since it was readily detectable using either a Src family-specific peptide (Fig. 3A) or enolase (Fig. 3B). Various stimuli, including thrombin (38), growth factors (39), and stretch (40) were shown to induce translocation of Src to the cytoskeleton or complex formation between the kinase and other molecules. These events may decrease the availability of Src by the specific antibody. However, we could not detect any significant difference in the total amount of Src protein immunoprecipitated from iso- or hypertonic samples (Fig. 3B), and no change was observed in the amount of Src associated with the Triton X-100-insoluble (cytoskeletal) extracts obtained after iso- or hypertonic treatment (Fig. 3D). These findings show that the decreased activity was due to reduced specific activity of the kinase. Moreover, the time course of the effect showed that the inhibition was rapid, clearly detectable after 1 min, arguing against the possibility that transient activation was followed by a longer lasting inhibition (Fig. 3C).

In subsequent experiments, we tested whether the hypertonicity-induced Src inhibition is caused by a decrease in cell volume per se or is due to other concomitantly changing parameters, such as osmolarity, pH, or other intracellular ion concentrations. Shrinkage activates NHE-1, causing a sizable cytosolic alkalization (≥0.4 pH unit; see below) in cells expressing this isoform. Since acidification of the cytoplasm was
reported to stimulate Src in kidney cells (23, 41), it was conceivable that an NHE-1-mediated cytosolic alkalinization might contribute to the inhibition of the kinase. To test this assumption, the effect of hypertonicity was investigated in the presence of HOE 694, a very potent and specific inhibitor of NHE-1. Fig. 4A shows that HOE 694 alone had no significant effect (caused a minimal inhibition) on Src activity. The drug, however, had a strong influence on the hypertonicity-induced Src response; in HOE-treated cells, the osmotic challenge not only failed to inhibit Src, but it caused an approximately 50% increase in its activity. Moreover, hypertonic exposure of NHE-3 cells did not decrease but rather moderately increased the activity of precipitated Src (data not shown). These findings are consistent with a role for NHE-1 in participating in the hypertonicity-induced inhibition of Src, presumably through the ensuing cytoplasmic alkalinization. In keeping with this notion, in the absence of functional NHE-1, shrinkage is known to induce metabolic acidification (15), which might stimulate Src (23). To address whether changes in pHi can in fact regulate Src activity in CHO cells, we manipulated pHi under iso-osmotic conditions using ionophores. Intracellular alkalinization was achieved by the Na+/H+ exchange ionophore monensin, whereas acidification was induced by the K+/H+ exchanger nigericin. As determined in parallel fluorimetric experiments, a 2.5-min treatment of the cells with monensin or nigericin caused an approximately 0.3-unit rise or 0.6-unit drop in the pHi, respectively (not shown). As shown in Fig. 4B, monensin caused a significant decrease (~40%), whereas nigericin induced a substantial increase (~60%) in Src activity. Thus, Src activity appears to be sensitive to pH changes in both directions, and this phenomenon is likely to contribute to the hypertonic inhibition of the kinase. To test whether Src can be regulated by the volume change itself, we used nystatin-permeabilized cells where shrinkage can be induced without any accompanying change in pHi, and in other intracellular monovalent ion concentrations. For these experiments, cells were kept in a KCl-based (intracellular-like) medium, in the presence of nystatin. To prevent swelling of the permeabilized cells (in which the colloid-osmotic pressure of the intracellular proteins is not counterbalanced) 60 mM sucrose was also included in the medium. As shown earlier, this procedure ensures the maintenance of the resting cell volume (12). Shrinkage was induced by the addition of an extra 300 mM sucrose. Fig. 4B shows that in the permeabilized cells a decrease in the volume did not reduce Src activity, but instead it led to a modest yet reproducible stimulation of the kinase. Taken together, these findings suggest that hypertonicity may influence Src activity by a complex mechanism both through changes in pHi and in the cell volume. However, the overall effect of hypertonicity on Src under conditions where increased protein tyrosine phosphorylation and cortactin phosphorylation are readily detectable is inhibitory.

Fyn kinase has also been implicated in the phosphorylation of cortactin (29, 34). To test whether hypertonicity might differentially regulate this kinase, its activity was also determined in immunoprecipitates. Data shown in Fig. 5 summarizes the effect of hypertonicity on Fyn. 10-min hypertonic treatment caused an approximately 2.9- or 1.7-fold increase in Fyn activity, using the Src family-specific peptide or enolase as substrate, respectively (Fig. 5, A and B). PP1 (0.5 μM) added to the kinase reaction mixture reduced Fyn activity by 90% (not shown). Hyperosmotic shock elicited an increase in the in vitro

Fig. 3. The effect of hyperosmolarity on the activity of p60SRC kinase. NHE-1 cells were treated with isotonic solutions or challenged with hypertonicity for 10 min (A and B) or for the indicated times (C). Thereafter, cells were lysed with radioimmune precipitation (A) or Triton buffer (B and C) and subjected to immunoprecipitation using a monoclonal anti-Src antibody. Immunocomplex kinase assays were performed using [γ-32P]ATP and either a peptide fragment of Cdc2 (A) or enolase (B and C) as substrate (see “Experimental Procedures” for details). The peptide was separated by phosphocellulose filters, and the incorporated activity was measured by scintillation counting (n = 4, reflecting the Src activity of cell lysates containing 120 μg of protein). When enolase was used, the samples were subjected to SDS-polyacrylamide gel electrophoresis followed by radiography, and phosphorylation was quantified by a PhosphorImager. No radioactive bands were detected if the primary antibody or enolase was omitted from the reaction (not shown). Data are expressed as percentage change, compared with the isotonic activity (100%). Where error bars are indicated, the results are mean ± S.E. for 3–6 independent determinations. The amount of Src in the immunoprecipitates (B) and Triton X-100-insoluble (Tx) fractions (D) obtained from iso- and hypertonicity treated cells were determined by immunoblotting with anti-Src. In four repeated experiments, no significant difference was observed between the iso- and hypertonic samples.
FIG. 4. The involvement of NHE-1-mediated intracellular alkalinization in the inhibition of Src kinase. **A**, NHE-1 cells were pretreated with Iso-Na for 10 min, and (where indicated) the medium was supplemented with 15 μM of the NHE inhibitor HOE 694 for the last minute of the preincubation. Subsequently, the medium was exchanged to Iso- or Hyper-Na, with or without the drug, and after 2.5 min the cells were lysed with Triton buffer. *In vitro* kinase assays and the quantification of enolase phosphorylation (n = 3) were carried out as in Fig. 3. **B**, after a 10-min pretreatment with Iso-Na, the medium was replaced with Iso-Na without any ionophore (Control) or with Iso-Na supplemented with either 10 μg/ml monensin (Alkalinization (mon)) or 10 μg/ml nigericin (Acidification (nig)). The cells were lysed after 2.5 min, and the lysates were processed for the Src assay (n = 3). **C**, after a short pretreatment in Iso-Na, cells were briefly washed with the permeabilization medium and then were permeabilized under isovolemic conditions using the same solution supplemented with 400 units/ml nystatin and 60 mM sucrose. After 7 min, the medium was aspirated and replaced either by the same medium (Isovolumic) or by the permeabilization buffer containing an extra 300 mM sucrose (Shrunken). 10 min later, the cells were lysed, and the samples were processed for the immunocomplex Src kinase assays (n = 3).

FIG. 5. Hypertonicity activates p59^{fyn}. Cells were treated with iso- or hypertonic solutions for 10 min or for the indicated times and lysed. Fyn was immunoprecipitated from the extracts with a monoclonal antibody, and its activity was determined essentially as described for Src under Fig. 4. **A**, the Cdc2 peptide was used as substrate (n = 4, reflecting the Fyn activity of cell lysates containing 270 μg of protein), whereas in **B**, **C**, and **E**, enolase was applied (for **B** and **E**, n = 5). **D**, Fyn immunoprecipitates obtained from iso- and hypertonic samples were subjected to electrophoresis, blotted onto nitrocellulose, and probed with anti-phosphotyrosine (top). The same blot was stripped and reprobed with anti-Fyn (bottom). **E**, cells were permeabilized as detailed in the legend to Fig. 4 and were treated either with the permeabilization buffer ensuring isovolemia (I) or with this buffer supplemented with 300 mM extra sucrose, inducing shrinkage (S). After 10 min, cells were lysed and processed for the Fyn activity determination.
autophosphorylating activity of the kinase as well (Fig. 5C). To test whether the rise in Fyn activity might be associated with a hypertonicity-induced change in the tyrosine phosphorylation of this kinase itself, we probed Fyn immunoprecipitates with anti-phosphotyrosine. The level of tyrosine phosphorylation was significantly higher in Fyn obtained from the hypertonic than from the isotonic samples, while no change was detected in the amount of precipitated protein (Fig. 5D). This phenomenon was present in NHE-3 cells as well, suggesting that the Fyn tyrosine phosphorylation was not due to shrinkage-induced alkalinization (not shown). To verify that Fyn was in fact stimulated by hypertonicity through a decrease in cell volume, we used nystatin-permeabilized cells. As demonstrated on Fig. 5E, under these conditions cell shrinkage led to similar stimulation of the kinase as observed in non-permeabilized cells (Fig. 5, B and E). Taken together, a decrease in cell volume stimulates the auto- and heterokinase activity of Fyn, suggesting that this enzyme might be involved in the signal transduction of osmotic stress.

Comparison of Hypertonicity-induced Cortactin Tyrosine Phosphorylation in Wild Type, Src−/−, and Fyn−/− Fibroblasts—Several different mechanisms were reported to participate in the increased phosphorylation of various Src substrates. Besides elevated kinase activity, these include the recruitment of the kinase and the substrate in the same compartment (37, 42) and the noncovalent modification of the substrate, which increases the affinity of the kinase toward it (43, 44). For example, the Src-mediated phosphorylation of gelsolin and other actin-binding proteins was dramatically augmented by phosphatidylinositol 4,5-bisphosphate and related molecules (43). Interestingly, hypertonicity was shown to increase the level of inositol phosphates (45), and cortactin contains a phosphatidylinositol 4,5-bisphosphate binding site (46). Thus, the decreased in vitro Src activity does not rule out the potential involvement of this kinase in cortactin phosphorylation. Moreover, while the hypertonic activation of Fyn is certainly consistent with a role of this kinase in cortactin phosphorylation, it does not indicate that Fyn is the only enzyme mediating this reaction.

In order to further investigate the potential contribution of Src and Fyn in the in situ phosphorylation of cortactin, we utilized mouse fibroblast lines derived from wild type (WT) or Src– or Fyn-deficient animals (21). To verify the absence of the respective kinases and to assess whether a compensatory increase occurred in the expressed phosphorylated proteins, we immunoprecipitated Src and Fyn from each cell line (Fig. 6A). As expected, the WT cells expressed both proteins, whereas the products of the disrupted genes were entirely missing from the corresponding cell lines. Interestingly, we found that the Src−/− cells overexpressed Fyn, while Src expression of the Fyn−/− cells was similar to the WT. The different cells were subjected to iso- or hypertonic treatment, and cortactin was precipitated and probed for tyrosine phosphorylation (Fig. 6B). The expression of cortactin was similar in each cell line. Hypertonicity caused marked cortactin phosphorylation in the WT cells, and usually an even stronger response was detected in Src−/− (but Fyn-overexpressing) cells. This finding clearly shows that Src kinase is not required for mediating this osmotic response. In contrast, in Fyn−/− cells, the hypertonyicitc elicited cortactin phosphorylation was substantially weaker than in the WT. These results suggest that Fyn significantly contributes to osmotic phosphorylation of cortactin. On the other hand (at least in Src-containing cells), the presence of Fyn is not an absolute requirement.

The Effect of PP1 on the Hyperosmotic Stimulation of NHE-1 and Inhibition of NHE-3—Having shown that the Src kinases are responsive to changes in cell volume, we asked whether shrinkage can regulate NHE-1 and NHE-3 in CHO cells by activating members of the Src family. To address this question, we measured the osmotic responses of these antiporters in control and PP1-treated cells loaded with the fluorescent pH indicator BCECF. In NHE-1 cells (Fig. 7, A and B), hypertonicity caused a sizable alkalinization (≥0.4 pH unit), as reported earlier (15). Pretreatment of the cells with PP1 had no effect on their basal pH, and failed to prevent the hypertonicity-induced response; neither the rate nor the extent of the alkalinization was different in drug-treated and in control cells. Thus, the osmotic stimulation of NHE-1 does not seem to depend on the activation or activity of Src kinases. Next, we performed experiments to discern whether this enzyme family can be involved in the hypertonic inhibition of NHE-3 (Fig. 7, C and D). To this end, control or PP1-treated NHE-3 cells were acidified, and the sodium-induced recovery of their pH was monitored under iso- or hypertonic conditions in the absence or presence of the drug. Hypertonicity strongly reduced the rate and the amplitude of the recovery in agreement with earlier reports by us and others (15, 17). PP1 did not affect the rate of the sodium-induced alkalinization under isotonic conditions and failed to influence the inhibition of NHE-3 by hypertonicity. These findings suggest that, at least in CHO cells, the Src family of tyrosine kinases does not appear to play an essential role in the hyperosmotic inhibition of NHE-3.

**DISCUSSION**

Cortactin Tyrosine Phosphorylation: Possible Mechanisms and Significance—An important finding of the present work is
Fig. 7. The Src family inhibitor PP1 does not affect the hyperosmotic stimulation of NHE-1 and hyperosmotic inhibition of NHE-3 in CHO cells. CHO cells expressing NHE-1 (A and B) or NHE-3 (C and D) were grown to confluence on glass coverslips, serum-deprived for 3 h, and loaded with the fluorescent pH indicator, BCECF, as detailed under “Experimental Procedures.” A, dye-loaded NHE-1 cells were bathed in Iso-Na medium without or with 10 μM PP1 for 10 min, and their basal fluorescence was recorded. When indicated by the arrow (HYPER), the medium was rapidly exchanged to Hyper-Na, in the absence (CONT) or presence of the drug (PP1). At the end of each run, the fluorescence response was calibrated in terms of pH, (see “Experimental Procedures”). B, the initial rate of the hypertonicity-induced alkalization in the absence (CONT) or presence of the drug (PP1) is shown (n = 4). C, NHE-3 cells were loaded with BCECF in Iso-Na medium with or without PP1. In order to acidify them, the cells were treated with NH₄Cl in Iso-, or Hyper-Na. Where indicated, PP1 was present during this period as well. The cells were then washed with Iso- or Hyper-Na solutions, their basal pH was recorded, and recovery was initiated (at the arrow marked Na⁺) by superfusion with Iso- or Hyper-Na, in the presence or absence of PP1, as shown by the traces. D, rates of recovery at the various pH values were determined as the slope of the linear line fitted to each 0.1 pH segment of the pH traces. H⁺ flux values were calculated by multiplying the rate of pH recovery by the buffering capacity previously determined throughout the pH range studied (15). Data are expressed as means ± S.E., for 3–6 determinations for each condition.

the identification of the cortical actin-binding protein, cortactin as one of the major targets of the hyperosmotic shock-induced tyrosine phosphorylation in CHO cells. We provide evidence that cortactin phosphorylation is due to a decrease in cell volume and is independent of the hypertonicity-provoked changes in pH or in the concentration of major cytoplasmic ionic constituents. Our observation that the Src family inhibitor, PP1, abolishes the hypertonicity-induced phosphorylation of many proteins, including cortactin, suggests that this kinase family plays an important role in the shrinkage-related protein phosphorylation in general and in cortactin phosphorylation in particular. Specifically, Fyn kinase appears to be one of the enzymes responsible for the osmotic stress-induced cortactin phosphorylation. This notion is supported by our findings that (a) hypertonicity stimulates Fyn and (b) the osmotically induced cortactin phosphorylation is substantially mitigated in Fyn-deficient cells. The presence of Fyn, however, is not an absolute requirement, implying the involvement of other kinases. Another tyrosine kinase that has been suggested to directly phosphorylate cortactin in erythroleukemia cells is Syk (47). Interestingly, this enzyme can also be stimulated by hyperosmotic stress (48). However, it is unlikely that Syk is involved in cortactin phosphorylation in CHO cells because, in agreement with earlier findings (49), we could not detect any immunoreactive Syk in CHO cells, and the Syk inhibitor piceatannol was not effective in preventing the hypertonicity-provoked cortactin phosphorylation (data not shown). On the other hand, we propose that the hyperosmotic stimulation of Syk in blood cells can be due to Src family-mediated Syk phosphorylation. In favor of this notion, Src has been shown to phosphorylate Syk (50), the hyperosmolarity-induced Syk phosphorylation is not due to autophosphorylation (51), and in neutrophils the hypertonic Syk phosphorylation can be prevented by PP1.² Another candidate is FER kinase, which was recently suggested to be involved in growth factor-induced cortactin phosphorylation (52). Future studies should define whether FER is also a volume-sensitive enzyme that may participate in the phenomenon. Direct kinase activation, however, may not be the only mechanism that accounts for the phosphorylation. Cortactin has recently been shown to translocate to the plasma membrane after growth factor stimulation (53). Our preliminary data suggest that cell shrinkage may also induce cortactin redistribution to the cell periphery (not shown). Such relocalization could facilitate phosphorylation by targeting the molecule to the vicinity of membrane-associated Src kinases.

Cortactin is thought to be involved in the organization of the cortical actin skeleton, in the regulation of cell-cell contact, cell motility, and tumor invasiveness (54). While the role of its tyrosine phosphorylation has not been entirely clarified, certain cytoskeletal changes have been associated with it. In Src-transformed cells, cortactin is primarily localized in podosomes, i.e. abnormal contact sites between the membrane and the substratum (28). In vitro phosphorylation of cortactin by

² A. Kapus, K. Szászi, J. Sun, S. Rizoli, and O. D. Rotstein, unpublished observation.
Src caused a strong down-regulation of its actin cross-linking activity (55). In keeping with this, Csk-deficient cells, in which cortactin is hyperphosphorylated, have a reduced number of stress fibers (30). These changes are reminiscent of the alterations in the cytoskeleton observed during hypotonic stress. For example, in yeast, osmotic shock induces the rapid disassembly of the actin cables, followed by a reassembly in the cortical region (6). This process is similar to the cycle of stress fiber disassembly/reassembly observed in fibroblasts, after the stimulation of Src (56). Since actin-cross-linking proteins of the cortical skeleton were found to be necessary for protection against osmotic shock in Dictyostelium (57), it is tempting to speculate that dynamic reorganization of cortactin (perhaps its redistribution from the stress fibers to the membrane skeleton) may also serve a similar role. Our future studies are directed to characterize the shrinkage-induced alteration of the cytoskeleton in mammalian cells and to assess the role of cortactin in this process.

The Participation of Src Kinases in Stress Signaling and Volume Regulation—Members of the Src family have been reported to participate in the signaling of oxidant (58) and UV-induced cellular stress (59). This study demonstrates that the two most widely expressed members of the family, Src and Fyn, are responsive to osmotic stress as well. The involvement of the individual members of the family in the signaling of oxidative and osmotic stress is selective. For example, in the same Src-deficient fibroblasts, where the oxidative shock-induced phosphorylation of big mitogen-activated protein kinase is completely abolished (58), the osmotic shock-promoted cortactin phosphorylation remains intact. We found that in CHO cells, hypertonicity decreased Src and increased Fyn activity. The most plausible explanation for this differential behavior is that hypertonicity not only alters cell volume but also activates NHE-1, and the consequent alkalinization may selectively modify Src activity. This notion is supported by our findings that (a) the presence of a functional NHE-1 is required for the hypertonic induction of Src and (b) cytosolic alkalinization inhibits, whereas acidification stimulates, Src (see also Refs. 23 and 41) even under iso-osmotic conditions. These observations raise the possibility that NHE-1 may be involved in the regulation of Src. It must be emphasized, however, that the NHE-mediated pH change may not be the only mechanism, and the contribution of other factors such as a rise in intracellular Na⁺ is also possible. When only the volume can change, both Src and Fyn are stimulated by hypertonicity, although the effect on Src is very modest. In neutrophils, Hck and Fgr were stimulated, whereas Lyn was inhibited, by hypertonicity (13). Further studies should clarify whether the opposite behavior can also be ascribed to the combined pH and volume effects.

The emerging picture is that cell shrinkage and swelling affects specific Src kinases, which can modify various volume-regulating ion transport processes. Swelling has been recently reported to stimulate Lek, which in turn phosphorylates and activates chloride channels leading to regulatory volume decrease (60). In erythrocytes, the absence of Hck and Fgr augmented the activity of potassium/chloride cotransport, a swelling-stimulating pathway (61). Rather surprisingly, however, the osmotic regulation of a major volume-transporter, NHE-1, does not seem to be mediated by Src kinases. The hypothesis correlating hypertonicity-induced, presumably Src family-mediated tyrosine phosphorylation and the activation of NHE-1 was derived from observations made in neutrophils (13). Since these cells express a number of hematopoietic cell-specific Src members, it was conceivable that in neutrophils certain Src kinases might play an important role in hypertonic NHE-1 activation. However, we regard this possibility as unlikely, because PP1, which has a dramatic inhibitory effect on the hypertonic tyrosine phosphorylation also in neutrophils, failed to affect the hypertonicity-triggered alkalinization in this cell type as well (data not shown). These observations suggest that the vast majority of the hypertonicity-induced tyrosine phosphorylation and NHE-1 activation are parallel but not causally related processes.

While Src has been shown to control the expression of NHE-3 (41), apparently it does not participate in the acute osmotic regulation of this transporter either. The role of two other osmosensitive kinase pathways (p38 and MEK/extracellular signal-regulated kinase) was also ruled out on pharmacological grounds (62). Since inhibitor studies suggest the involvement of tyrosine phosphorylation in the osmotic control of the antiporters, other kinases must be considered. A potential candidate is the Jak/Stat pathway which was, recently found to be stimulated by osmotic stress (63).

The mechanism by which hypertonicity affects Src kinases or the NHE isoforms is unknown. Shrinkage was shown to induce clustering of cell surface receptors (64), and this process may turn on many signaling pathways. It is conceivable that common upstream events lead to the activation of Src type kinases and other kinases and initiate signaling toward NHE. Such common factor might be the potential osmotic activation of small G-proteins. It is noteworthy that Rac and Cdc42 were suggested to play a role in the osmotic shock-induced activation of stress kinases (65), and their activation by growth factors was shown to induce membrane translocation of cortactin (53). Furthermore, Rho has been implicated in NHE regulation (66) and was found to stimulate myosin light chain phosphorylation (67), a reaction that seems to be necessary for the osmotic activation of NHE-1 (68). Future studies should test the intriguing possibility that small G proteins may be upstream mediators of osmotic shock.

In summary, the present studies show that ubiquitous Src kinases are regulated by cell volume, that cortactin is a major target of hypertonicity-induced phosphorylation and that Fyn kinase is involved in this reaction. The Src family may represent an important link between cell volume and the organization of the cytoskeleton, whereas other kinase pathways convey the message to the Na⁺/H⁺ exchanger.

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