Hyperosmotic stress induced by treatment of Swiss 3T3 cells with the non-permeant solutes sucrose or sorbitol, rapidly and robustly stimulated endogenous focal adhesion kinase (FAK) phosphorylation at Tyr-397, the major autophosphorylation site, and at Tyr-577, within the kinase activation loop. Hyperosmotic stress-stimulated FAK phosphorylation at Tyr-397 occurred via an Src-independent pathway, whereas Tyr-577 phosphorylation was completely blocked by exposure to the Src family kinase inhibitor PP-2. Inhibition of p38 MAP kinase or phosphatidylinositol 3-kinases did not prevent FAK phosphorylation stimulated by hyperosmotic stress. Overexpression of N17 RhoA did not reduce hyperosmotic stress-mediated localization of phosphorylated FAK to focal contacts and treatment with the Rho-associated kinase inhibitor Y-27632 did not prevent FAK translocation and tyrosine phosphorylation in response to hyperosmotic stress. Overexpression of N17 Rac only slightly altered the hyperosmotic stress-mediated localization of phosphorylated FAK to focal contacts. In contrast, overexpression of the N17 mutant of Cdc42 disrupted hyperosmotic stress-stimulated FAK Tyr-397 localization to focal contacts. Additionally, treatment of cells with Clostridium difficile toxin B potently inhibited hyperosmotic stress-induced FAK tyrosine phosphorylation. Furthermore, FAK null fibroblasts compared with their FAK containing controls show markedly increased sensitivity, manifest by subsequent apoptosis, to sustained hyperosmotic stress. Our results indicate that FAK plays a fundamental role in protecting cells from hyperosmotic stress, and that the pathway(s) that mediates FAK autophosphorylation at Tyr-397 in response to osmotic stress can be distinguished from the pathways utilized by many other stimuli, including neuro peptides and bioactive lipids (Rho- and Rho-associated kinase-dependent), tyrosine kinase receptor agonists (phosphatidylinositol 3-kinase-dependent), and integrins ( Src-dependent).

A successful response to hyperosmotic stress is of fundamental importance to cell survival as evidenced by the remarkable conservation of cellular osmotic stress response pathways from yeast to humans. In the budding yeast, Saccharomyces cerevisiae, exposure to a hyperosmotic environment leads to activation of a well characterized signaling cascade comprising cell surface osmosensors, Sho1p and Sln1p/Ypd1p/Ssk1p, mitogen-activated protein kinase cascade, Ste11 or Ssk2p (MAP3Ks),1 Pbs2p (MAPKK), and Hog1p (MAPK). Activation of this pathway results in the nuclear translocation of Hog1p, and finally to expression of genes leading to a survival response (1–8). In mammalian cells, exposure to hyperosmotic stress also stimulates p38 MAP kinase (mammalian Hog1p homologue), but prolonged exposure activates SAPK/JNK and ultimately leads to apoptosis (9, 10). In some cell types, this apoptotic response to prolonged hyperosmolar stress involves increased cytosolic Ca2++ ([Ca2+]i) (9, 11) triggering activation of the FAK-related non-receptor tyrosine kinase, Pyk2 (also known as CAK/ RAPTK), which can be activated by increased [Ca2+]i (10). Many mitogenic and/or anti-apoptotic signaling molecules, including receptor tyrosine kinases (12), the Src family of non-receptor tyrosine kinases (13), the “AGC” (protein kinases A, C, and G) subfamily of protein kinases (14, 15), and the p21-activated kinases (16) are stimulated in mammalian cells by exposure to transient hyperosmotic stress. Indeed, all three MAP kinase families, p38 MAPK, SAPK/JNK, and ERK1/2 are activated in mammalian cells in response to hyperosmotic stress (17, 18). Hyperosmotic stress also induces rapid cortical actin remodeling (19) and importantly, activation of all three Rho-GTPase family members, Rho, Rac, and Cdc42 in different cell types (20, 21). Although prolonged exposure to hyperosmotic stress ultimately leads to apoptosis (9, 22), little is known about which of these pathways may act to counteract hyperosmotic stress-stimulated apoptosis in mammalian cells.

FAK is a non-receptor tyrosine kinase first identified as one of many proteins phosphorylated on tyrosine in v-Src transformed chicken embryo fibroblasts (23). FAK has been shown to be a critical point of convergence in the action of multiple

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1 The abbreviations used are: MAP3K, mitogen-activated protein kinase kinase; DMEM, Dulbecco's modified Eagle's medium; FAK, focal adhesion kinase;GPCR, G-protein coupled receptor; Ab, antibody; PI 3-kinase, phosphatidylinositol 3-kinase; PP-2, pyrazolo pyrimidine 2; ROK, Rho-associated kinase; SAPK, stress-activated protein kinase; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 1% Tween 20; IEC-18, rat intestinal epithelial cells; mAb, monoclonal antibody; TBS, Tris-buffered saline; HA, heamagglutinin; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; CBD, Cdc42-binding domain; CAS, CT 10-regulated kinase-associated substrate.
signaling pathways initiated by integrins (24–28), onecogenic forms of Src (29), G protein-coupled receptor (GPCR) agonists, including mitogenic neuroepetides (30–34) and bioactive lipids (35–37), bacterial toxins (38, 39), and growth factors (40–43). FAK is rapidly phosphorylated on multiple tyrosines, including Tyr-397, the major autophosphorylation site, Tyr-576 and Tyr-577 (within the FAK kinase activation loop), and Tyr-861 and Tyr-925 in cells stimulated by extracellular ligands. The biological importance of FAK is underscored by the fact that FAK−/− knockout animals are not viable, exhibiting a defect in mesodermal development and cells that showed impaired locomotion (44). FAK activation has also been shown to block p53-mediated anoikis when epithelial cells are deprived of their extracellular matrix attachments (45). It has recently been demonstrated that FAK and Pyk2, although structurally related, are differentially regulated in many systems (46), and their functions have been shown in some instances to be mutually antagonistic (47). The potential role of FAK in anti-apoptotic signaling in response to hyperosmotic stress has not been examined.

In the present study, we demonstrate that exposure to su- crose or sorbitol leading to hyperosmotic stress rapidly and robustly stimulates FAK phosphorylation at Tyr-397, the maj or autophosphorylation site, and at Tyr-577, within the kinase activation loop, in Swiss 3T3 cells. The results presented in this study imply that FAK autophosphorylation at Tyr-397 in response to osmotic stress is mediated through a pathway(s) that can be distinguished from the pathways utilized by neuropep tides and bioactive lipids (Rho- and ROK-dependent), tyrosine kinase agonists (PI 3-kinase-dependent) and integrins (Src-re dependent). In contrast, overexpression of the N17 mutant of Cdc42 disrupted hyperosmotic stress-stimulated localization of FAK phosphorylated at Tyr-397 to focal contacts. Furthermore, treatment of Swiss 3T3 cells with Cdcslidium dificile toxin B potently inhibits hyperosmotic stress-induced FAK Tyr-397 phosphorylation. Additionally, we show that FAK−/− fibroblasts derived from FAK null embryos are markedly more susceptible to sustained hyperosmotic stress exposure, undergo increasing apoptosis compared with FAK expressing fibroblasts exposed for equal lengths of time. Src-null cells also exhibit similar susceptibility to hyperosmotic stress-induced apoptosis compared with c-Src-expressing cells. These results indicate a novel signaling cascade leading to FAK/Src activation in Swiss 3T3 cells in response to hyperosmotic stress and support the idea that the FAK and c-Src participate in protect ing mammalian cells from hyperosmotic stress.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Stock cultures of Swiss 3T3 cells were maintained in DMEM, supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO2 and 90% air at 37 °C. For experimental purposes, Swiss 3T3 cells were plated in 100-mm dishes at 6 × 10^6 cells/dish in DMEM supplemented with 10% fetal bovine serum and used after 6–8 days when cells were confluent and quiescent. Stock cultures of IEC-18 (rat ileal epithelial cells) were maintained in DMEM supplemented with 5% fetal bovine serum, and passed every 4–5 days (never allowed to become fully confluent). For experiments, IEC-18 cells were plated in 100-mm dishes at 6 × 10^6 cells/dish in DMEM supplemented with 5% fetal bovine serum and used after 6–8 days when cells were confluent. FAK null (FAK−/−) and FAK expressing (FAK+/-) control fibroblasts (44) were maintained in DMEM with 4 mM l-glutamine adjusted to contain 0.5% d-glucose and 4.5% l-glutamate, 90%; fetal bovine serum, 10%, in a humidified atmosphere containing 10% CO2 and 90% air at 37 °C. For experiments, 1 × 10^6 cell/35-mm dish were plated and experiments were performed at 5 days (90% confluence). SYP cells (CRL-2459) and YF cells (CRL-2497) (49) were maintained in DMEM with 4 mM l-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate and 4.5 g/liter glucose, 95%; fetal bovine serum, 5%, in a humidified atmosphere containing 10% CO2 and 90% air at 37 °C. For experiments, 1 × 10^6 cell/35-mm dish were plated and experiments were performed at 5 days (90% confluence).
Cdc42-GTP Pull-down Assay—Confluent, cultures of IEC-18 cells were serum starved overnight, and washed with warm serum-free DMEM 30 min before starting stimulation. Cells were then incubated in DMEM in the presence or absence of 0.45 M sucrose for 30 min at 37 °C. After which the cells were rapidly washed 1 time in PBS at room temperature, and lysed in ice-cold lysis buffer containing, 50 mM Tris, pH 7.5, 10 mM MgCl₂, 0.3 mM NaCl, and 2% IGEPA (polyoxyethylene nonylphenol) supplemented with 1× protease inhibitor mixture (Cytoskeleton, Inc., Denver, CO). Lysates were clarified by centrifuging for 5 min at 8,000 rpm in a 4 °C microcentrifuge. Samples of clarified lysates were saved for loading controls. 20 μl of glutathione-agarose beads, to which a fusion protein, glutathione S-transferase-CBD (Cdc42-binding domain), of WASP was bound (Cytoskeleton, Inc.) and 10 μl of 100× protease inhibitor mixture (Cytoskeleton, Inc.) were added to each of the freshly obtained clarified lysates and the mixture was incubated at 4 °C on a rotator for 1 h. Beads were pelleted by centrifugation at 5,000 × g for 3 min at 4 °C. The pelleted beads were then washed in ice-cold wash buffer containing, 25 mM Tris, pH 7.5, 30 mM MgCl₂, 40 mM NaCl. The washed beads were then resuspended in 20 μl of 4× SDS-PAGE sample buffer (400 mM Tris-HCl, pH 6.8, 2 mM EDTA, 12% SDS, 8% 2-mercaptoethanol, 20% glycerol), boiled 10 min, and the attached protein, along with the saved clarified lysates, were analyzed by SDS-PAGE. Western blots were performed as in the above protocol (membrane corresponding to molecular weights below 97,000) with anti-Cdc42 primary antibody (Cytoskeleton, Inc.) or (membrane corresponding to molecular weights above 97,000) with anti-FAK-Tyr(P)-397 Ab (0.1 μg/ml).

Apoptosis Assay—FAK−/−, FAK+/−, SYF, or YF cells were plated at 1 × 10⁵ cell/35-mm dish and after 5 days in culture (90% confluent) they were washed 2 times in DMEM, then exposed to DMEM alone or DMEM containing 0.45 M sucrose for increasing times at 37 °C, after which they were washed 3 times with DMEM. They were then incubated for 24 h after which they were fixed in phosphate-buffered formalin (Fisher) for 30 min followed by permeabilization with TBS with 0.1% Triton X-100 for 5 min. Cells were then washed in TBS and incubated in FITC-labeled dUTP and dNTP in reaction buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 1 mM CoCl₂, 0.25 mg/ml bovine serum albumin, pH 6.6; Roche Diagnostics GmbH) for 60 min at 37 °C in a humidified incubator. Following terminal deoxynucleotide transferase reaction, cells were washed 3 times in TBS, then imaged immediately (FAK−/− and FAK+/− cells) or further stained with a 1:5000 dilution of 4,6′-diamidino-2-phenylindole (Invitrogen Molecular Probes, Inc.) for 30 min at room temperature, followed by a further washing 3 times in TBS. Cells were imaged with an epifluorescence microscope (Zeiss Axioskop) with a Zeiss water immersion objective (Achromplan 40/0.75 w, Carl Zeiss, Inc.) under FITC fluorescence and DIC settings (FAK−/− and FAK+/− cells), or under FITC fluorescence and 4,6′-diamidino-2-phenylindole settings (SYF and YF cells) and photographed with a CCD camera. Images were processed using Adobe Photoshop CS.

Statistical Analysis of Apoptosis Quantification—Five high powered fields were photographed per experiment and the FITC positive fragmented nuclei were counted as were the total number of cells/field over 900 total cells were counted/experimental group). Statistical significance was determined by a one-tailed Student's t test as described by Jekel et al. (50).

Materials—Bombesin, sucrose (ultra-pure), and sorbitol (ultra-pure) were all obtained from Sigma. Horseradish peroxidase-conjugated donkey anti-rabbit antibody (NA 934) and sheep anti-mouse antibody and ECL reagents were from Amersham Biosciences. PY-20 anti-phosphotyrosine mAb was from ICN, and the 4G10 anti-phosphotyrosine mAb was from UBI, Lake Placid, NY. Anti-paxillin and anti-CAS monoclonal antibodies were obtained from Signal Transduction Laboratories. Anti-FAK antibody C20 was from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal phospho-specific Abs to FAK Tyr-397, FAK Tyr-577, or total FAK (C20) were obtained from BIOSOURCE International (Camarillo, CA). PP-2 and PP-3 were obtained from Calbiochem-Novabiochem. cDNA plasmids encoding influenza-HA-tagged L17N mutants of RhoA, Rac1, and Cdc42 were obtained from Guthrie DNA resource center, Guthrie Research Institute, Sayre, PA. All other reagents used were of the highest grade available. CBD-WASP beads, anti-Cdc42 polyclonal antibody, wash, and lysis buffers for Cdc42 pull-down assay all obtained from Cytoskeleton, Inc.
ulation with 5 mM bombesin or 20% fetal bovine serum, shown for comparison. Immunoprecipitation with anti-FAK followed by Western blot with the same antibody (lower blot) shows that recovery of FAK from cell lysates is not altered by hyperosmotic treatment. The results shown in Fig. 1A demonstrate that hyperosmotic stress by either sucrose or sorbitol induces FAK tyrosine phosphorylation in Swiss 3T3 cells.

Many stimuli, including integrin clustering by fibronectin, and GPCR stimulation by bombesin and other agonists, lead to FAK tyrosine phosphorylation at Tyr-397 (autophosphorylation site) and FAK Tyr-577 (one of two Src phosphorylation sites within the FAK activation loop). Consequently, we examined whether these sites were phosphorylated in response to hyperosmotic stress stimulation. Confluent and quiescent Swiss 3T3 cells were exposed to 0.45 M sucrose at 37 °C for increasing times. Cell lysates were analyzed by SDS-PAGE and Western blot using antibodies that detect the phosphorylated state of FAK tyrosine 397 (FAK Tyr(P)-397), or tyrosine 577 (FAK Tyr(P)-577). Fig. 1B shows representative blots probed for FAK Tyr(P)-397 or FAK Tyr(P)-577. Hyperosmotic stress-stimulated FAK phosphorylation is observed by 5 min, peaks at 30 min, and is sustained for at least 60 min. These results demonstrate that hyperosmotic stress stimulates FAK phosphorylation on Tyr-397 and Tyr-577, in a rapid and sustained manner.

**CAS and Paxillin Tyrosine Phosphorylation Are Also Stimulated by Hyperosmotic Stress**—The stimulation of FAK tyrosine phosphorylation by many stimuli is coordinated with the tyrosine phosphorylation of other focal adhesion proteins including p130Cas (CAS) and paxillin (51, 52). We examined whether hypertonic stress also induces tyrosine phosphorylation of CAS and paxillin. Quiescent Swiss 3T3 cells were incubated in media containing 0.45 M sucrose or 0.6 M sorbitol for 10 min at 37 °C and then lysed. The cell extracts were incubated with anti-phosphotyrosine-specific mAb and the immunoprecipitates were analyzed by Western blot with anti-CAS or anti-paxillin antibodies. Fig. 1C shows that hyperosmotic stress also stimulated the tyrosine phosphorylation of CAS and paxillin within 1 min of exposure and this phosphorylation peaked by 30 min.

To substantiate that CAS and paxillin become phosphorylated on tyrosine residues in response to hyperosmotic stress, quiescent Swiss 3T3 cells were incubated in media containing 0.45 M sucrose for various times at 37 °C. Cell lysates were immunoprecipitated with anti-CAS mAb (Fig. 1D) or anti-paxillin mAb (not shown), and the immune complexes were analyzed by SDS-PAGE followed by Western blot with anti-phosphotyrosine mAb (4G10). The kinetics of tyrosine phosphorylation as demonstrated by immunoprecipitating with the CAS antibodies followed by Western blot with phospho-specific antibodies (Fig. 1D), were identical to those shown in Fig. 1C. We also confirmed that exposure of cells to another non-permeant solute, sorbitol, also increases CAS and paxillin tyrosine phosphorylation (not shown).

To determine whether hyperosmotic stress-stimulated FAK tyrosine phosphorylation is mediated by cell shrinkage or by intracellular hypertonicity (53), we compared the FAK tyrosine phosphorylation stimulated in quiescent Swiss 3T3 cells by incubation in media containing membrane-impermeable 0.45 M sucrose or 0.6 M sorbitol, with that stimulated by incubation in media containing 0.45 M urea. Urea is membrane-impermeable and thus causes both intra- and extracellular hypertonicity without changing intracellular osmotic pressure and consequently it does not cause cell shrinkage. We found that hyperosmolar urea did not lead to increased tyrosine phosphorylation of FAK, paxillin, or CAS (not shown), suggesting that the osmotic stress-stimulated tyrosine phosphorylation of these proteins is caused by differential osmotic pressure-mediated cell shrinkage and not by intracellular hyperosmolarity per se (13, 54). These results demonstrate that hyperosmotic stress stimulates a rapid and pronounced increase in the tyrosine phosphorylation of FAK, CAS, and paxillin.

**Role of p38 in Hyperosmotic Stress-stimulated FAK Activation**—Next, we examined the mechanism(s) by which hyperosmotic stress leads to tyrosine phosphorylation of focal adhesion proteins. Numerous studies in yeast demonstrate the central role of the HOG1 mitogen-activated protein kinase in survival of hyperosmotic stress. The mammalian homologue of Hog1, p38 MAPK, is also activated by osmotic stress. Using a mAb against dually phosphorylated Thr-180/Tyr-182 p38 MAP kinase, we confirmed that exposure of Swiss 3T3 cells to hyperosmotic stress stimulates the activation of p38 MAP kinase to levels comparable with those induced by ultraviolet exposure, and in a time-dependent fashion (Fig. 2A, upper panel).

![FIG. 2. Role of p38 MAP kinase (p38) and PI 3-kinase in hyperosmotic stress-induced FAK tyrosine phosphorylation. A, upper panel, kinetics of p38 activation by hyperosmotic stress. Confluent, quiescent Swiss 3T3 cells were incubated with 0.45 M sucrose for increasing times at 37 °C and lysed with 4× SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and immunoblotted with polyclonal antibody to p38 MAP kinase (see “Experimental Procedures”). The autoradiogram shown is typical of results obtained in five independent experiments. B, role of PI 3-kinase in hyperosmotic stress-induced FAK tyrosine phosphorylation. Confluent and quiescent Swiss 3T3 cells were preincubated for 60 min with 0, 0.3, or 3 mM concentrations of SB202190 or SB203580 followed by 15 min incubation in the presence or absence of 0.45 M sucrose, and subsequently lysed with 4× SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and immunoblotted with anti-phosphorylated FAK Tyr(P)-397 and anti-dually phosphorylated p38 MAP kinase. The p38 blots were stripped and reblotted with anti-p38 MAP kinase for control. Results shown here are characteristic of results obtained in five independent experiments. C, role of PI 3-kinase in hyperosmotic stress-induced FAK tyrosine phosphorylation. Confluent and quiescent Swiss 3T3 cells were preincubated for 60 min at 37 °C in the presence or absence of 30 mM LY294002, then incubated in 0.45 M sucrose for increasing times, and subsequently lysed in 4× SDS-PAGE sample buffer. Samples were divided equally, analyzed by SDS-PAGE, and immunoblotted with anti-phosphorylated FAK Tyr(P)-397 (FAK pTyr-397) and FAK Tyr-577 (FAK pTyr-577). The phospho-FAK Tyr-577 blot was then stripped and reblotted with total FAK antibody. The autoradiograms shown are typical of results obtained from three independent experiments.

![Image]
confirmed that pretreatment with either SB203580 or SB202190, selective p38 MAP kinase inhibitors, blocked hyperosmotic stress-induced p38 activation (lower panel). Fig. 2A, lower panel, also shows that treatment with either SB202190 or SB203580, at concentrations that blocked p38 activation by hyperosmotic stress did not produce any effect on FAK Tyr-397 phosphorylation. We conclude therefore, that hyperosmotic stress stimulation of FAK tyrosine phosphorylation is not mediated by p38 MAP kinase activation.

Similarly, we examined the role of PI 3-kinase, which has been implicated in osmo-sensing, on FAK tyrosine phosphorylation stimulated by hyperosmotic stress. Fig. 2B shows that preincubation with 30 μM LY29004, a selective inhibitor of PI 3-kinase activity, inhibited neither FAK Tyr-397 nor FAK Tyr-577 phosphorylation, induced by exposing Swiss 3T3 cells to 0.45 M sucrose for either 5 or 30 min. Similar results were obtained when wortmannin (50 μM) was used instead of LY29004. These results suggest that neither p38 MAP kinase nor PI 3-kinase activities are necessary for hyperosmotic stress-induced rapid activation of FAK tyrosine phosphorylation.

Role of Src Family Kinase Activity in Hyperosmotic Stress-stimulated FAK Tyrosine Phosphorylation—To confirm results of others (55, 56), showing that hyperosmotic stress activates Src family kinases, confluent, quiescent Swiss 3T3 cultures of these cells were incubated in media containing 0.45 M sucrose for increasing times. Fig. 3A shows rapid and sustained hyperosmotic stress activation of Src as judged by Western blotting with an antibody that detects phosphorylated Src at tyrosine residue 418 (autophosphorylation site reflecting active kinase).

Integrin stimulation of FAK phosphorylation both at Tyr-397 and Tyr-577 requires Src kinase activity, whereas only GPCR-induced stimulation of FAK Tyr-577 phosphorylation is Src-dependent (57, 58). GPCR-induced FAK Tyr-397 does not require Src and probably occurs by autophosphorylation. We, therefore, examined the Src family dependence of hyperosmotic stress on specific FAK tyrosine phosphorylation sites (Tyr-397 and Tyr-577), using the selective inhibitor of Src family kinases, pyrazolopyrimidine (PP-2). At 10 μM, PP-2 completely inhibits Src, but not FAK, kinase activity (59, 60). Swiss 3T3 cells were pretreated with 10 μM PP-2 or the same concentration of PP-3, a structurally related but inactive analogue of PP-2, or DMEM alone for 30 min. Pretreatment was followed by incubation in media containing 0.45 M sucrose or 5 nM bombesin for 15 min at 37 °C. Cell lysates were immunoprecipitated with anti-FAK Ab, and the immune complexes were examined by SDS-PAGE followed by Western blotting with a phosphorytosine-specific antibody. The supernatants from the first immunoprecipitation were re-immunoprecipitated with phosphorytosine-specific antibody and these immunoprecipitates were examined by SDS-PAGE followed by Western blotting with anti-paxillin mAb. As shown in Fig. 3B (upper panel), the tyrosine phosphorylation of FAK stimulated by hyperosmotic stress is attenuated by Src family kinase inhibition. Fig. 3B (lower panel) also shows inhibition of paxillin tyrosine phosphorylation induced by hyperosmotic stress in cells treated with PP-2. These results suggest that Src family kinase activity is required, at least in part, for hyperosmotic stress-stimulated FAK tyrosine phosphorylation.

Integrin-mediated activation of FAK is intimately linked to the stimulation of non-receptor tyrosine kinases of the Src family (reviewed in Ref. 61). When cells are re-plated onto fibronectin, FAK Tyr-397 phosphorylation is dependent on Src kinase activity. Src also phosphorylates FAK on tyrosines 577 and 576, within the activation loop of the kinase, further increasing its catalytic activity. However, the initial event in GPCR-stimulated FAK activation by bombesin or lysophosphaticid acid, namely FAK Tyr-397 phosphorylation, is not dependent on Src kinase activity (57), whereas subsequent GPCR-stimulated Tyr-P)-577 and Tyr-P)-576 are Src-dependent events. We sought to determine the role of Src in the phosphorylation of specific FAK tyrosine residues in Swiss 3T3 cells exposed to hyperosmotic stress. Initially, we examined the Src kinase dependence of hyperosmotic stress-stimulated FAK Tyr-397 phosphorylation. Fig. 3C (upper panel) demonstrates that hyperosmotic stress promotes FAK phosphorylation at Tyr-397 (lanes 2–4) even in the presence of PP2 (lanes 5–7). In contrast, Fig. 3C (middle panel) demonstrates FAK Tyr-577 phosphorylation induced by hyperosmotic stress (lanes 2–5) is strikingly inhibited by pretreatment with PP2 (lanes 5–7) of Swiss 3T3. 

**Fig. 3. Role of Src kinase activity on hyperosmotic stress-induced FAK tyrosine phosphorylation.** A, kinetics of Src activation induced by hyperosmotic stress. Confluent and quiescent Swiss 3T3 cells were incubated for increasing times at 37 °C in 0.45 M sucrose and lysed in 4× SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and immunoblotted with antibody against phosphorylated Src tyrosine 418 (Anti-Src pTyr-418). The autoradiogram shown is typical of results obtained in four independent experiments. B, effect of Src kinase inhibition on hyperosmotic stress-induced total FAK phosphorylation. Confluent and quiescent Swiss 3T3 cells were preincubated for 30 min in the presence or absence of PP2 or PP3 followed by incubation for 15 min in 0.45 M sucrose, and subsequent lysis with RIPA buffer as described under “Experimental Procedures.” Lysates were immunoprecipitated with anti-total FAK antibody followed by reimmunoprecipitation with monoclonal anti-FAK tyrosine antibody. The FAK immuno precipitates were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine monoclonal antibody (Anti-PY) followed by membrane stripping and reblotting with total FAK antibody (Anti-FAK). The phosphorytosine immuno precipitates were analyzed by SDS-PAGE and immunoblotted with monoclonal anti-paxillin antibodies. Autoradiograms shown are typical of results obtained in four independent experiments. C, effect of Src kinase inhibition on FAK Tyr-397 and FAK Tyr-577 phosphorylation induced by hyperosmotic stress. Confluent and quiescent Swiss 3T3 cells were preincubated for 30 min in the presence or absence of PP2, then incubated for increasing times in 0.45 M sucrose and then lysed in 4× SDS-PAGE sample buffer.
cells. Fig. 3C suggests that FAK Tyr-397 autophosphorylation induced by hyperosmotic stress is Src-independent, whereas subsequent phosphorylations including Tyr-577, are Src-dependent. Thus, our results demonstrate that hyperosmotic stress-induced phosphorylation of FAK Tyr-397, the autophosphorylation site of this kinase, is not dependent on the function of p38 MAP kinase, PI 3-kinase, or Src in Swiss 3T3 cells.

Hyperosmotic Stress Induces Both Assembly of Focal Contacts and F-actin Remodeling—FAK phosphorylation at Tyr-397 induced by multiple stimuli in Swiss 3T3 cells is associated with re-localization of FAK from the cytosol to focal complexes. The assembly of these structures is directed by Rho-GTPases, which play a fundamental role in promoting distinct organizations of the actin cytoskeleton. Osmotically induced actin remodeling has been found in various systems (19–21, 62, 63).

We examined the subcellular localization of phosphorylated FAK Tyr-397 and F-actin organization using the FAK Tyr-397 phospho-specific antibody and TRITC-phalloidin, respectively, in sparse, serum-starved (Fig. 4, left two panels), bombesin-treated (Fig. 4, middle two panels), or sucrose-treated (Fig. 4, right two panels) Swiss 3T3 cells. As illustrated in Fig. 4 (left panels) very few focal adhesions or stress fibers were detected in serum-starved cells. Exposure of cells to bombesin or sucrose led to a marked increase in the localization of autophosphorylated FAK to focal contacts but in different distributions. A comparison of the middle and right panels in Fig. 4 indicates that hyperosmotic stress induced new central fine, thread-like actin fibers, small central focal contacts (64), and peripheral short filopodial-like actin extensions terminating in focal contacts all containing FAK Tyr(P)-397 (64, 65), as opposed to the large, RhoA-mediated, well defined focal adhesions and dense parallel arrays of bundled actin stress fibers induced by bombesin stimulation.

In multiple cell types, the Rho family of GTPases mediate morphological changes in actin organization and in the size and spatial arrangement of focal complexes (64, 65), concomitantly rearranging the localization of focal adhesion proteins such as FAK to specific cell-extracellular matrix contact structures. Recent studies using proximal tubule epithelial (LLC-PK1) cells demonstrate that Rho and its downstream effector, Rho-associated kinase (ROK), are activated by hyperosmotic stress (21). Rho and ROK have previously been shown by our laboratory and others to play a central role in FAK Tyr-397 phosphorylation stimulated by the GPCR agonist bombesin and lysophosphatidic acid. We therefore examined the role of RhoA and its downstream effector ROK in hyperosmotic stress-induced FAK Tyr-397 phosphorylation.

To elucidate the role of RhoA in this process we overexpressed a HA-tagged T17N mutant of RhoA into sparsely plated Swiss 3T3 cells. T17N RhoA is defective in binding GTP and demonstrates increased affinity for RhoGEFs and consequently acts as a dominant negative mutant. Following transfection, the cells were cultured for 48 h, serum-starved for a further 2 h and then placed in 0.45 M sucrose for 30 min. Cells were then fixed and co-stained using anti-FAK phospho-Tyr-397 Ab and anti-HA primary Abs followed by appropriate fluorescent-labeled secondary antibodies. The typical immunofluorescence photomicrographs shown in Fig. 5A illustrate that all cells were equivalently labeled with anti-FAK-phospho-Tyr-397 (visualized on left), whereas cells differentially stained for HA indicated differential expression of dominant negative RhoA (visualized on right). Comparison of the distribution of FAK phosphorylated at Tyr-397 in untransfected cells versus ones overexpressing T17N RhoA after 30 min hyperosmotic stress shows little consequence of the dominant negative GTPase on localization and amounts of visualized FAK Tyr-397 phosphorylation. However, this same N17 RhoA construct was able to inhibit the formation of actin stress fibers induced by bombesin in these cells (not shown). This result, combined with the distinct hyperosmotic stress-induced actin cytoskeletal changes presented in Fig. 4, suggested that, at least in Swiss 3T3 cells, RhoA was not necessary for initiating FAK Tyr-397 phosphorylation.

To substantiate the results obtained with T17N RhoA, we next examined the role of the downstream effector of Rho, ROK. We have shown that preincubation of Swiss 3T3 cells with 10 μM Y27632, a selective inhibitor of ROK, markedly abrogates bombesin-simulated phosphorylation of FAK at Tyr-397, and stress fiber formation and assembly of focal adhesions (66). Confluent, quiescent Swiss 3T3 cells were preincubated with 10 μM Y27632 for 30 min followed by 15 min in 0.45 M sucrose. As shown in Fig. 5B, the low level of basal phosphorylation of FAK Tyr-397 was further diminished by ROK inhibition, but the prominent increase in FAK Tyr-397 phosphorylation...
FIG. 5. Role of RhoA and Rho-associated kinase in HS induction of FAK tyrosine phosphorylation. A, RhoA is not required for hyperosmotic stress-induced rearrangement of phosphorylated FAK Tyr-397 (anti-FAK pTyr-397) and anti-HA (anti-HA) antibodies. Photomicrographs shown are representative of fields containing both transfected and untransfected cells taken from transfection performed in at least three independent experiments. B, ROK inhibition does not inhibit the hyperosmotic stress induced increase in FAK Tyr-397 phosphorylation. Confluent and quiescent Swiss 3T3 cells were preincubated for 30 min in the presence or absence of 10 μM Y27632 after which they were incubated a further 15 min in 0.45 M sucrose and lysed in 4X SDS-PAGE sample buffer. All samples were analyzed by SDS-PAGE and immunoblotted with phospho-FAK Tyr-397 antibody (FAK pTyr-397). The blot was then stripped and rebotted with total FAK antibody (FAK total). The autoluminogram shown here is representative of results obtained in three independent experiments. C, ROK inhibition does not affect the redistribution of phosphorylated FAK Tyr-397 induced by hyperosmotic stress. Sparse cultures of Swiss 3T3 cells were preincubated in the presence or absence of 10 μM Y27632 followed by a further 30 min in 0.45 M sucrose. Cells were subsequently fixed and stained with anti-FAK phospho-tyrosine 397 followed by Alexa Fluor 488-conjugated secondary antibody. Cells were visualized and photographed using a x40 water immersion lens and Zeiss Axioskop microscope. Photomicrographs are representative of typical cells under the specific conditions performed in four independent experiments.

To examine whether Rac1 or Cdc42 play a role in hyperosmotic stress-stimulated FAK tyrosine phosphorylation, we also overexpressed HA-tagged T17N (dominant negative) mutants of Rac1 and Cdc42 in sparsely plated Swiss 3T3 fibroblasts. After 48 h, the cells were serum-starved for 2 h followed by exposure to 0.45 M sucrose for 30 min. Cells were then fixed and stained, as described under “Experimental Procedures,” simultaneously for the HA-tagged mutant GTPases and for phosphorylated FAK Tyr-397. The distribution pattern of FAK Tyr(P)-397 is shown in Fig. 6A (upper panels) in N17 Rac1-transfected cells, after 30 min osmotic stress, was slightly altered in comparison to untransfected neighboring cells, with cells still maintaining their shape but forming larger focal complexes. Fig. 6A (lower panels) shows confocal fluorescence photomicrographs of typical N17 Cdc42-transfected cells alongside an untransfected cell. Interestingly, N17 Cdc42 expression strikingly prevented the hyperosmotic stress-induced re-localization of FAK Tyr(P)-397 to focal contacts. In contrast, FAK Tyr(P)-397 localized to thin central and peripheral focal complexes in neighboring untransfected cells. The results illustrated in Fig. 6A strongly suggest a central role for Cdc42 in hyperosmotic stress-mediated phosphorylation of FAK on Tyr-397.

The effect of C. difficile Toxin B—To substantiate that Cdc42 mediates FAK tyrosine phosphorylation stimulated by hyper-osmotic stress, in cells pretreated with or without 10 μM Y27632 after which they were incubated a further 15 min in 0.45 M sucrose and lysed in 4X SDS-PAGE sample buffer. All samples were analyzed by SDS-PAGE and immunoblotted with phospho-FAK Tyr-397 antibody (FAK pTyr-397). The blot was then stripped and rebotted with total FAK antibody (FAK total). The autoluminogram shown here is representative of results obtained in three independent experiments. C, ROK inhibition does not affect the redistribution of phosphorylated FAK Tyr-397 induced by hyperosmotic stress. Sparse cultures of Swiss 3T3 cells were preincubated in the presence or absence of 10 μM Y27632 followed by a further 30 min in 0.45 M sucrose. Cells were subsequently fixed and stained with anti-FAK phospho-tyrosine 397 followed by Alexa Fluor 488-conjugated secondary antibody. Cells were visualized and photographed using a x40 water immersion lens and Zeiss Axioskop microscope. Photomicrographs are representative of typical cells under the specific conditions performed in four independent experiments.

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Effect of Expressing Dominant Negative Rac1 and Cdc42 on Hyperosmotic Stress-stimulated FAK Tyrosine Phosphorylation—In addition to Rho activation, hyperosmotic stress leads to the activation of the two other well characterized members of the Rho family of small GTPases, namely Rac1 and Cdc42 (20, 67).
FIG. 6. Role of Rac1 and Cdc42 in phosphorylated FAK Tyr-397 redistribution induced by hyperosmotic stress. A, N17 Rac1 and N17 Cdc42 expression, effects on cell shape and FAK Tyr(P)-397 redistribution in response to hyperosmotic stress. Sparse cultures of Swiss 3T3 cells were transfected with a vector encoding HA-tagged N17 Rac1 (top two epifluorescence photomicrographs) or HA-tagged N17 Cdc42 (bottom two confocal photomicrographs), 48 h post-transfection cells were incubated in 0.45 M sucrose for 30 min. Cells were subsequently fixed in phosphate-buffered formalin and stained with both anti-phosphorylated FAK Tyr-397 (visualized in left panels top and bottom) and anti-HA antibodies (visualized in right panels top and bottom). Photomicrographs were taken focusing at the level of the dish for N17 Rac1-transfected cells. However, because of marked cell rounding in N17 Cdc42-transfected cells confocal images were obtained as described under “Experimental Procedures.” Photomicrographs are of representative collections of transfected and untransfected cells and are typical of results obtained from three independent experiments. B, C. difficile toxin B inhibits FAK tyrosine phosphorylation induced by hyperosmotic stress. Confluent and quiescent cultures of Swiss 3T3 cells were preincubated in the presence or absence of 30 μg/mL C. difficile toxin B for 3 h. They were then incubated at 37 °C in 0.45 M sucrose for the times indicated and lysed in 4× SDS-PAGE sample buffer. All samples were analyzed in duplicate by SDS-PAGE and immunoblotted with anti-phospho-FAK Tyr-397 (FAK pTyr-397) or anti-phospho FAK Tyr-577 (FAK pTyr-577). Blots from FAK-Tyr-577 were then stripped and reblotted with total anti-FAK antibody (Total FAK). Shown here is a representative autoradiogram where identical results were obtained in four independent experiments. C, hyperosmotic stress induces GTP loading on Cdc42, and stimulates phosphorylation of FAK tyrosine 397 in IEC cells. Confluent cultures of IEC-18 cells were serum starved overnight. They were then incubated for 30 min in DMEM in the presence or absence of 0.45 M sucrose. Lysates were clarified and 40-μL aliquots were boiled for 10 min in sample buffer. GTP-loaded Cdc42 was pulled down by incubation with glutathione-agarose beads coated with CBD-WASP-glutathione S-transferase fusion protein. The precipitates were then analyzed by SDS-PAGE along side the previously sampled lysate aliquots. Analysis with Western blotting with antibodies to FAK Tyr(P)-397, and Cdc42 was performed. The FAK blot was then stripped and rebotted with anti-total FAK antibody. C, FAK shows FAK Tyr(P)-397 (FAK pTyr-397) levels in IEC-18 cells in control (−) and after 30 min with 0.45 M sucrose (suc). Total FAK levels (Total FAK) are shown in the lower blot for comparison. Cdc42 shows levels of Cdc42-GTP pulled down by CBD-WASP-beads (CBD-WASP pull-down) and the total Cdc42 levels in the original lysates for comparison (lower panel) from IEC-18 cells under control (−) and after 30 min with 0.45 M sucrose (suc).
osmotic stress exposure, we examined the effect of inhibiting the function of Rho family GTPases using C. difficile toxin B. This toxin, a member of a family of "large" clostridial toxins, glucosylates specific threonines within the effector region of Rho, Rac, and Cdc42 thereby blocking their interactions with effector proteins (68). Quiescent Swiss 3T3 cells were pre-treated for 3 h with this toxin at 10 ng/ml, followed by 0.45 M sucrose for increasing times. Fig. 6A shows that phosphorylation of FAK, either on Tyr-397 (upper panel) or Tyr-577 (middle panel), induced by hyperosmotic stress is completely blocked by treatment with C. difficile toxin B, without affecting the total amount of FAK loaded (Fig. 6B, bottom panel). Results presented here, excluding significant participation of RhoA, ROK (Fig. 5), and Rac1 (Fig. 6A), when taken with the dramatic inhibition of hyperosmotic stress-induced FAK Tyr-397 phosphorylation by preincubation with C. difficile toxin B, strongly suggest the involvement of Cdc42 in this novel pathway of FAK activation.

Hyperosmotic Stress Induces Activation of FAK and Cdc42 in Intestinal Epithelial Cells—To extend our results in fibroblasts, on FAK Tyr-397 phosphorylation and Cdc42, we next turned to IEC-18 cells (derived from rat intestinal epithelial crypts (48), reasoning that gastrointestinal tract epithelial cells experience at least transient hyperosmotic stress, and as such are physiologically relevant cells to study in this context. Confluent, quiescent cultures of IEC-18 cells were serum starved overnight, after which they were incubated a further 30 min in serum-free DMEM in the presence or absence of 0.45 M sucrose. Cells were lysed, lysates clarified, and Cdc42GTP pull-down was performed with CBD (Cdc42-biding domain) of WASP-coated agarose beads ("Experimental Procedures"). CBD-WASP precipitates were analyzed by SDS-PAGE alongside aliquots of the original clarified lysates (prior to pull-downs). After transferring to polyvinylidene difluoride membranes, Western blots were performed separately with anti-Cdc42 antibody and with total FAK antibody followed by stripping and reblotting with anti-FAK Tyr(P)-397 antibodies. As seen in Fig. 6C, we detected Cdc42GTP in lysates from IEC-18 cells exposed to 0.45 M sucrose for 30 min, but not from control IEC-18 cells (lower panel, Cdc42GTP), whereas the total Cdc42 levels were unchanged (lower panel, total Cdc42). The upper panel of Fig. 6C shows that although the basal levels of phosphorylated FAK at Tyr-397 are high in these cells, hyperosmotic stress induces a significant increase in FAK Tyr(P)-397 (upper panel, FAK Tyr(P)-397), whereas total FAK (upper panel, Total FAK) levels are unchanged. The results shown in Fig. 6C suggest, first that hyperosmotic stress induction of FAK Tyr-397 phosphorylation also occurs in epithelial cells, and second that hyperosmotic stress increases the formation of Cdc42 loaded with GTP.

Physiological Significance of FAK in the Response to Hyperosmotic Stress—The above experiments show experimental evidence for signaling pathways by which FAK is phosphorylated on Tyr-397 and Tyr-577 in response to exposure to hyperosmotic stress, however, the physiologic role FAK might be playing in the mammalian cell response to hyperosmotic stress is unclear. Given that FAK overexpression blocks anoikis (69) and prevents UV-stimulated apoptosis (70), we hypothesized that FAK might also play a protective role in the mammalian cell response to hyperosmotic stress. We decided to test this hypothesis by taking advantage of the paired fibroblast cell lines, (FAK+/+ and FAK−/−) developed by Ilic et al. (71). We exposed 90% confluent, overnight serum-starved FAK+/+ cells and FAK−/− cells to DMEM alone or to 0.45 M sucrose for increasing times. After the hyperosmotic stress exposure period, cells were washed thoroughly in DMEM and incubated a further 18 h, at which time they were fixed and assayed for apoptosis by TUNEL assay, as described under “Experimental Procedures.” As shown in Fig. 7, basal levels of apoptosis in both FAK+/+ and FAK−/− were not significantly different. In contrast, a 60-min exposure to 0.45 M sucrose induced a dramatic increase in TUNEL positive cells in FAK−/− (38%) as compared with their FAK+/+ (7%) counterparts after the same stress exposure. Extended exposure (24 h) to hyperosmotic stress, however, did result in both FAK+/+ and FAK−/− cells to undergo virtually 100% apoptosis (not shown). We conclude that FAK is playing a protective role in the mammalian cell response to transient hyperosmotic stress.

**Triple Src Family Null, SYF Cells Show Increased Susceptibility to Hyperosmotic Stress-induced Apoptosis Compared with c-Src Expressing YF Cells**—The close functional relationship
FIG. 8. Src-expressing (YF) cells, when compared with Src-null (SYF) cells, are markedly more resistant to transient hyperosmotic stress. A, apoptosis, assayed by TUNEL, induced in SYF cells by hyperosmotic stress is greater than that induced in YF cells. 90% confluent, serum-starved cultures of SYF and YF fibroblast were incubated in DMEM alone (Control No HS) or in DMEM with 0.45 M sucrose for 60 min followed by washing and further incubation in DMEM alone. Cell were fixed in phosphate-buffered formalin and a TUNEL assay was performed using FITC-labeled dUTP, as described in the legend to Fig. 7. Cells were then counterstained using DAPI (see “Experimental Procedures”) to assay total cell number. Shown in the upper panels are fluorescent photomicrographs (showing FITC-labeled nucleotides incorporated into apoptotic nuclei), from YF and SYF cell cultures after incubation in DMEM alone (control no HS) for 24 h, or DMEM with 0.45 M sucrose for 60 min (60 min HS) followed by washing in DMEM and further incubation for 23 h in DMEM. The corresponding DAPI images (showing total cell nuclei) are shown in the panel immediately below. B, quantification of apoptosis. Three separate experiments were performed and paired fluorescence and DIPI images were obtained in at least 11 fields/condition. The % of TUNEL positive cells was obtained for each of the 5 fields (over 1000 cells evaluated per condition for each experiment). The graph shown shows a comparison of % apoptosis in SYF and YF cells after incubation in DMEM alone (control no HS) for 24 h, or DMEM with 0.45 M sucrose for 60 min (60 min HS) followed by washing in DMEM and further incubation for 23 h in DMEM. Columns indicated with "#" sign were statistically different with p < 0.001.

between FAK and c-Src, and their coincident activation by hyperosmotic stress shown in this study (Figs. 1 and 3), prompted us to investigate whether c-Src also plays a role in protecting mammalian cells from hyperosmotic stress. We took advantage of Src family triple null (Src, Yes, and Fyn null) SYF cells and c-Src expressing (Yes and Fyn null) YF cells (also known as Src-/- cells) (49). These cells only differ in that the YF cells express wild type levels of c-Src. SYF cells and YF cells were grown to 90% confluence and exposed for 60 min at 37 °C to DMEM (control), or DMEM containing 0.45 M sucrose. The cells were washed and apoptosis was assayed by TUNEL assay 23 h later (see “Experimental Procedures”). As shown in Fig. 8B, basal levels of apoptosis in SYF and YF cells are comparable (16.5 and 16.1%, respectively), however, hyperosmotic stress induced a dramatic increase in apoptosis in SYF cells (34.8%), whereas the proportion of YF cells undergoing apoptosis after exposure to 0.45 M sucrose (13.74%) remained virtually unchanged. We conclude that c-Src, like FAK also protects cells against transient hyperosmotic stress.

DISCUSSION

Hyperosmotic stress is a ubiquitously experienced environmental condition that cells have been required to withstand throughout evolution. It leads to dramatic cellular shape changes, but its effect on specific FAK tyrosine phosphorylation sites was unknown. Extensive work characterizing hyperosmotic stress-induced signaling pathways leading to changes in pro-survival gene transcription, has been done in the budding yeast, S. cerevisiae (1–7). In S. cerevisiae, the hyperosmotic stress-activated pathway, from the cell surface osmosensors, Sho1p and Sln1p/Ypd1p/Ssk1p, to nuclear translocation of Hog1p are well understood (8). It has also been established that S. cerevisiae hyperosmotic signal transduction requires proper cellular co-localization of Pbs2p and the Sho1p osmosensor and this co-localization requires the small GTPase, Cdc42p (72). Pro-survival hyperosmotic stress-mediated signaling pathways in mammalian cells have been much less extensively characterized. FAK has been shown to play a role in protecting mammalian cells from apoptosis resulting from loss of anchorage to extracellular matrix (69), and from ultraviolet irradiation (70) but to date no studies demonstrate its role in preventing apoptosis resulting from hyperosmotic stress. Results presented here demonstrate that FAK, CAS, and paxillin are shrinkage-sensitive proteins, and that exposure to hyperosmotic stress potently stimulates the phosphorylation of FAK at Tyr-397 and Tyr-577 in Swiss 3T3 cells. Additionally, we demonstrate a fundamental role for FAK in preventing mammalian cell apoptosis triggered by prolonged exposure to hyperosmotic stress.

The previously described studies from yeast have shown a prominent role for the p38 MAP kinase homologue, Hog1p, in hyperosmotic stress signaling. Hyperosmotic stress also potently activates p38 MAP kinase in animal cells, including Swiss 3T3 cells. The activation of p38 MAP kinase, through serine phosphorylation of Hsp27 (heat shock protein 27), can enhance actin remodeling and focal adhesion turnover, at least in some cell types (73). We therefore examined the requirement of p38 MAP kinase in hyperosmotic stress-stimulated FAK.
phosphorylation. Our results show that inhibitors of p38 MAP kinases, SB202190 and SB203580, at concentrations that completely blocked p38 MAP kinase activation, do not inhibit hyperosmotic stress-stimulated FAK tyrosine phosphorylation. Platelet-derived growth factor stimulation of membrane ruffles, assembly of focal contacts, and FAK tyrosine phosphorylation is mediated by stimulation of PI 3-kinase activity (40). Signaling by PI 3-kinases is mediated by 3-phosphoinositide binding to regulatory subunit pleckstrin homology domains. In Swiss 3T3 mouse fibroblasts, osmotic stress has been shown to increase both phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate (74). In the present study, we demonstrate that hyperosmotic stress-stimulated FAK tyrosine phosphorylation is not blocked by the presence of the selective PI 3-kinase inhibitors LY29004 or wortmannin, at concentrations that completely prevent platelet-derived growth factor-induced FAK tyrosine phosphorylation (41), implying that hyperosmotic stress stimulates FAK through a PI 3-kinase-independent pathway. These results show that transient exposure to hyperosmotic stress leads to FAK tyrosine phosphorylation through a PI 3-kinase-independent pathway.

It is known that exposure to hyperosmotic stress leads to increased tyrosine phosphorylation of multiple proteins in a variety of mammalian cell types (12, 55, 75). The Src family non-receptor tyrosine kinases have been shown to mediate the increase in the tyrosine phosphorylation of cortactin and other unidentified proteins induced by hyperosmotic stress (13, 56). One of the multiple mechanisms thought to be operative in Src kinase activation is a conformation switch promoted by high affinity binding of its SH2 domain to phosphorylated tyrosines within SH2 consensus sequences on other proteins, thereby releasing it from its inactive closed conformation that is maintained by autoinhibitory intramolecular binding to phosphorylated Tyr-527 (61, 76). Src kinase binding via its SH2 domain to phosphorylated Tyr-397 of FAK leads to Src kinase activation and Src-mediated tyrosine phosphorylation of FAK at additional sites, including Tyr-576 and Tyr-577 located in the activation loop of the kinase catalytic domain. Src phosphorylation of FAK at these sites is required for maximal FAK catalytic activity (77, 78). Hence, stimuli that activate FAK, can potentially stimulate the maximal activity of both FAK and Src kinases.

FAK activation stimulated by integrin clustering in cells plated on fibronectin requires Src kinase activity, both for the initial phosphorylation of FAK at Tyr-397, and for the phosphorylation of FAK at Tyr-576 and Tyr-577 (78). However, bombesin or lysophosphatidic acid, acting through endogenous GPCRs, stimulate FAK phosphorylation at Tyr-397 in a Src-independent manner (57). As hyperosmotic stress leads to Src family kinase activation, we investigated the requirement of Src family kinases in FAK tyrosine phosphorylation induced by hyperosmotic stress. Using the selective Src family kinase inhibitor PP-2, at concentrations previously shown to inhibit Src kinase activity, but not FAK activity (59, 60), we show that while Src kinase activity is needed for FAK Tyr-577 phosphorylation, it is not required for FAK Tyr-397 phosphorylation stimulated when Swiss 3T3 cells are exposed to hyperosmotic stress. These results indicate that hyperosmotic stress-stimulated FAK phosphorylation at Tyr-397 can be distinguished from that initiated by integrin engagement and it is most likely the result of autophosphorylation rather than of transphosphorylation mediated by Src.

FAK activating stimuli, including neuroptides via their GPCRs, polypeptide growth factors via their receptor tyrosine kinases, and fibronectin via integrins, all cause characteristic changes in cellular shape, actin cytoskeleton, and focal adhesions. These events, including actin remodeling and cellular shape changes, are mediated by members of the Rho family of small GTPases. Rho activation stimulates the assembly of parallel arrays of actin stress fibers and promotes the formation of well defined focal adhesions (79). Rac activation induces formation of lamellipodia and actin recruitment into membrane ruffles (79), whereas Cdc42 signaling leads to the formation of filopodia and membrane microspikes (65). Rac and Cdc42 induce the assembly of small focal adhesions termed focal contacts (64). Here we examined the potential role of known actin remodeling signaling pathways in FAK tyrosine phosphorylation stimulated by exposure to hyperosmotic stress.

Studies from our laboratory and others, demonstrated that GPCR stimulation of FAK tyrosine phosphorylation by neuroptides (bombesin) and bioactive lipids (lysophosphatidic acid) depends on RhoA, its downstream effector ROK, and subsequent F-actin reorganization (37, 51, 66, 80–83). Here we demonstrate that, in contrast to bombesin, hyperosmotic stress did not induce the formation of parallel arrays of actin stress fibers in Swiss 3T3 cells. Furthermore, neither expression of the dominant negative N17 RhoA mutant, nor treatment with the specific ROK inhibitor Y27632, prevented hyperosmotic stress-stimulated FAK tyrosine phosphorylation. These results indicate that the Rho-ROK pathway is not required for hyperosmotic stress stimulation of FAK tyrosine phosphorylation. Thus, our results indicate that the pathways that mediate FAK autophosphorylation at Tyr-397 in response to hyperosmotic stress can be distinguished from the pathways utilized by many other stimuli, including GPCR agonists such as neuroptides and bioactive lipids (Rho- and ROK-dependent), tyrosine kinase receptor agonists such as platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor (PI 3-kinase-dependent), and integrin activation by fibronectin (Src-dependent) (41, 42, 66, 81, 84, 85).

Previous studies in Swiss 3T3 cells have shown that microinjection of constitutively active Cdc42 promotes the formation of filopodia (64). Here we observed that hyperosmotic stress induced the formation of actin containing structures resembling filopodia (shown in Fig. 5, upper right panel) with small focal contacts containing FAK phosphorylated at Tyr-397. Furthermore, overexpression of the N17 (dominant-negative) mutant of Cdc42 disrupted the localization of FAK phosphorylated at Tyr-397 to focal contacts in response to hyperosmotic stress. In contrast, the overexpression of the N17 mutant of Rac did not prevent the localization of phosphorylated FAK to focal adhesions. A further salient feature of the results shown here is that hyperosmotic stress stimulated FAK phosphorylation at Tyr-397 was completely blocked by treatment with the pan-Rho-glucosylator C. difficile toxin B. Several lines of evidence outlined above suggest that Rho does not play a major role in mediating FAK tyrosine phosphorylation in response to hyperosmotic stress. Consequently, the results obtained with C. difficile toxin B implicate Cdc42 in mediating FAK tyrosine phosphorylation and relocalization to focal complexes induced by hyperosmotic stress. The induction of FAK Tyr-397 phosphorylation by hyperosmotic stress in IEC-18 cells suggests this response is one occurring in epithelial cell types as well. Our direct demonstration of Cdc42 activation in these cells also supports the notion that it plays a regulatory role in this stress survival pathway. The involvement of Cdc42 in hyperosmotic stress signaling implicated here is consistent with recent studies in neutrophils and Chinese hamster ovary cells (20), and with studies done in neuroblastoma cells implicating this Rho family GTPase in muscarinic cholinergic receptor-stimulated FAK tyrosine phosphorylation (86). Our results dem-
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...for the first time, the involvement of Cdc42 in hyperosmotic stress-induced FAK tyrosine phosphorylation in cultured fibroblasts is consistent with an evolutionary conserved role of Cdc42 in sensing and protecting against hyperosmotic stress.

Several studies have shown that FAK overexpression protects mammalian cells from apoptosis resulting from loss of anchorage to extracellular matrix (69), and from ultraviolet irradiation (70), but to date no studies demonstrate its role in hyperosmotic stress-induced apoptosis. Our results demonstrate that FAK protects mammalian cells from apoptosis caused by transient exposure to hyperosmotic stress.

The close functional relationship between FAK and Src and their coincident activation in response to hyperosmotic stress shown in this study, prompted us to consider that Src might also be required to protect cells against hyperosmotic stress. Indeed Src family tyrosine kinase activation protects mammalian cells from apoptotic promoting stimuli (87, 88), and the Src family kinase member Syk has been shown to protect the chicken DT40 B cell line from hyperosmotic stress-induced apoptosis (56). Our results indicate that c-Src protects mammalian cells from hyperosmotic stress-induced apoptosis.

In conclusion, we present here the supporting data that FAK and c-Src play a physiologic role in protecting mammalian cells from prolonged hyperosmotic stress, and a model of FAK tyrosine phosphorylation at specific sites induced by exposure of Swiss 3T3 mouse fibroblasts to hyperosmotic stress. Specifically, hyperosmotic stress-induced phosphorylation of FAK Tyr-397, via autophosphorylation, is regulated through the small Rho family GTPase, Cdc42, whereas the subsequent phosphorylation of FAK Tyr-576/Tyr-577(within the kinase activation loop) is mediated by Src kinase activity. Our results raise the attractive possibility that FAK activation via this novel pathway along with c-Src activation, contributes to the ability of mammalian cells to successfully withstand hyperosmotic stress.

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J. Adrian Lunn and Enrique Rozengurt

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