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Involvement of Integrins in Osmosensing and Signaling toward Autophagic Proteolysis in Rat Liver*

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Inhibition of autophagic proteolysis by hypoosmotic or amino acid-induced hepatocyte swelling requires osmosignaling toward p38^{MAPK}; however, the upstream osmosensing and signaling events are unknown. These were studied in the intact perfused rat liver with a preserved *in situ* environment of hepatocytes. It was found that hypoosmotic hepatocyte swelling led to an activation of Src (but not FAK), Erks, and p38^{MAPK}, which was prevented by the integrin inhibitory hexapeptide GRGDSP, but not its inactive analogue GRGESP. Src inhibition by PP-2 prevented hypoosmotic MAP kinase activation, indicating that the integrin/Src system is located upstream in the osmosignaling toward p38^{MAPK} and Erks. Inhibition of the integrin/Src system by the RGD motif-containing peptide or PP-2 also prevented the inhibition of proteolysis and the decrease in autophagic vacuole volume, which is otherwise observed in response to hypoosmotic or glutamine/glycine-induced hepatocyte swelling. These inhibitors, however, did not affect swelling-independent proteolysis inhibition by phenylalanine. In line with a role of p38^{MAPK} in triggering the volume regulatory decrease (RVD), PP-2 and the RGD peptide blunted RVD in response to hypoosmotic cell swelling. The data identify integrins and Src as upstream events in the osmosignaling toward MAP kinases, proteolysis, and RVD. They further point to a role of integrins as osmo- and mechanosensors in the intact liver, which may provide a link between cell volume and cell function.

Changes in cell hydration within a narrow, physiological range markedly affect carbohydrate and protein metabolism, hepatic bile flow, gene expression, and cellular susceptibility to environmental stress (1–6). The regulation of cell function by cell hydration requires structures, which sense hydration changes (osmosensing) and intracellular signaling pathways toward effector sites (osmosignaling).

Progress has been made in identifying signal transduction pathways linking cell volume changes to alterations in cell function (7). For example, the MAPK,[1] p38^{MAPK}, activation is critical for creating volume-regulatory ion fluxes in response to hypoosmotic swelling in perfused rat liver (8) and HTC liver cells (9). Likewise, proteolysis inhibition by cell swelling strongly depends on activation of the p38^{MAPK} in perfused rat liver (10). Specific inhibition of the p38^{MAPK} abolishes the antiproteolytic effects exerted by hypoosmolarity and glutamine, but is without effect on cell swelling under these conditions (10). Another component involved in swelling-dependent proteolysis inhibition is the microtubular system. Colchicine blocks hypoosmotic proteolysis inhibition, but not p38^{MAPK} activation upon hypoosmolarity (11). These data show that the microtubule-dependent element in hydration-dependent proteolysis signaling is obviously localized downstream of p38^{MAPK} activation. In contrast to agonists that cause changes of cell hydration, the antiproteolytic action of non-swelling amino acids, *e.g.* phenylalanine, resides on the activation of other signaling events, such as activation of mammalian target of rapamycin (mTOR) and p70 ribosomal S6 protein kinase (p70^{S6K} kinase) (12–14), which can clearly be differentiated from the swelling-related antiproteolytic signaling cascade (15).

Whereas in bacteria, plants, and fungi two-component histidine kinases were identified to be involved in sensing of and subsequent adaptation to adverse osmotic conditions (16), the mechanisms of osmosensing in mammalian cells are far from being understood. Integrins are candidates to be involved in mechanotransduction, *i.e.* the conversion of a mechanical stimulus into covalent modifications of signaling components. Integrins are heterodimers with each subunit having a single transmembrane domain. They establish cell adhesion to the extracellular matrix and bind inside the cell to cytoplasmic proteins, which in turn interact with different signal transduction components and the cytoskeleton (for reviews see Refs. 17 and 18). In normal liver, the most important integrins are $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_9\beta_1$ (19–21).

The present study investigates the role of integrins and Src in hypoosmotic signaling toward proteolysis inhibition and cell volume regulation in the isolated perfused rat liver, which most authentically represents the three-dimensional hepatocyte anchoring to the extracellular matrix, preserved cell polarity, intact cytoskeleton, and structural/functional cell-cell interactions. Using the integrin antagonistic peptide GRGDSP and the Src kinase inhibitor PP-2, an integrin-dependent activation of Src kinases was localized upstream of swelling-induced p38^{MAPK} signaling toward inhibition of autophagy. In contrast, the antiproteolytic effect of phenylalanine, which does not involve cell swelling and p38^{MAPK} does not depend on GRGDSP-sensitive integrin action and Src activation. Consistent with

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; AV, autophagic vacuole; DMEM, Dulbecco's modified Eagle's medium; Erk, extracellular signal-regulated kinase; FAK, focal adhesion kinase; MBP, myelin basic protein; PBS, phosphate-buffered saline; PI, phosphoinositide; RVD, regulatory volume decrease.

inhibition of osmosignaling toward $p38^{MAPK}$, GRGDSP and PP2 effectively antagonize the volume regulatory response triggered by hypoosmotic swelling. The findings suggest a role of integrins in hepatic osmosensing and transforming hepatocyte swelling into a physiological response.

EXPERIMENTAL PROCEDURES

Liver Perfusion—Livers from male Wistar rats (160–230 g), fed a standard diet, were perfused in an open non-recirculating manner at a flow rate of 3.5–4.5 ml/min/g. The perfusion medium was bicarbonate-buffered Krebs-Henseleit saline plus lactate (2.1 mmol/liter) and pyruvate (0.3 mmol/liter), incubated with O_2/CO_2 (19:1, v/v), at a temperature of 37 °C. For changing the osmolarity, the NaCl concentration (115 mmol/liter) was varied, resulting in corresponding changes of osmolarity. Additions were made either by the use of micropumps (20 μl/min) or by dissolution in Krebs-Henseleit buffer. Inhibitors were dissolved in Me_2SO. Viability of the livers was assessed by monitoring effluent oxygen concentration and measurement of lactate dehydrogenase leakage from livers, which did not exceed 15–20 milliunits/min/g of liver.

Monitoring and Assays in Liver Perfusion—Effluent perfusate pH was monitored continuously with a pH-sensitive electrode, and the perfusion pressure was detected by a pressure transducer (Hugo Sachs Electronics, Hugstetten, Germany). Basal portal pressure was 3–5 cm H_2O and was not affected by the compounds used in this study. The intracellular water space was calculated from the difference of washout profiles of simultaneously infused [^{14}C]urea and [^{3}H]inulin as described previously (22). In fed animals, the cell water under control conditions was 551 ± 10 μl/g (n = 28). Proteolysis was determined in separate perfusion experiments as ^{3}H label release from rats that had been injected intraperitoneally with 50 μCi of L-4,5-[^{3}H]leucine 16 h prior to the perfusion experiment as described previously (23). The rate of proteolysis was set to 100% under normotonic control conditions, due to different labeling of hepatic proteins after intraperitoneal injection, and the extent of inhibition of proteolysis was determined 30 min after institution of the respective condition, a time point, when a new steady state had been reached.

In bile experiments, livers were perfused with 100 μmol/liter [^{3}H]taurocholate (1 μCi/liter). Bile was collected at 2-min intervals. Bile flow was assessed by gravimetry, assuming a specific mass of 1 g/ml. Taurocholate excretion into bile was determined by liquid scintillation counting of the radioactivity present in bile, based on the specific radioactivity of [^{3}H]taurocholate in influent perfusate.

Preparation of Cultured Hepatocytes—Liver parenchymal cells were isolated from the livers of male Wistar rats (200 g body wt.) by collagenase perfusion as described previously by Meijer *et al.* (24). The cells were plated on fibronectin-coated culture dishes (17 μg/dish, diameter 60 mm, ~1 × 10^6 cells) and maintained in Krebs-Henseleit buffer (KHB) with 6 mmol/liter glucose, equilibrated in a humidified atmosphere (air/CO_2, 19:1, v/v) at 37 °C. After 4 h in KHB, the cells were cultured for another 48 h in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum and 1% penicillin/streptomycin, insulin (100 nmol/liter), 1% glutamine, dexamethasone (100 nmol/liter), sodium selenite (30 nmol/liter), and aprotinin (1 μg/ml). Fresh DMEM was added after 24 h. After a total cultivation time of 48 h, cells were cultured in normoosmotic DMEM without additions containing 1000 mg/liter glucose for 4 h. After starvation for 4 h in normoosmotic medium, cells were either exposed to hypoosmolar (205 mosmol/liter) or normoosmotic control medium (305 mosmol/liter) for 2 min. If indicated, cells were incubated with PP-2 (20 μmol/liter) or GRGDSP (250 μmol/liter) 20 min prior to installing hypoosmolarity or the normoosmotic control condition, respectively. At the end of experimental treatment, medium was removed from the culture, and cells were immediately lysed at 4 °C using lysis buffer containing 20 mmol/liter Tris-HCl (pH 7.4), 140 mmol/liter NaCl, 10 mmol/liter NaF, 10 mmol/liter sodium pyrophosphate, 1% Triton X-100, 1 mmol/liter EDTA, 1 mmol/liter EGTA, 1 mmol/liter sodium vanadate, 20 mmol/liter β-glycerophosphate, and protease inhibitor mixture (Roche Applied Science). The homogenized lysates were centrifuged at 20,000 × g at 4 °C, and protein analyses were performed as described below. Protein concentrations were determined according to Bradford (25).

Tissue Processing for Immune Complex Kinase Assays and Western Blot Analysis—Rat livers were perfused for 130 min with isoosmotic perfusion medium, thereafter with hypoosmotic perfusion medium (185 mosmol/liter). The desired osmolarity was achieved by omission of 60 mM NaCl. When indicated, inhibitors were present for 30 min prior to institution of hypoosmotic perfusion conditions or addition of amino acids. For immune complex assay and Western blot determinations, liver lobes from perfused liver were excised at the respective time points (0, 2, 5, 10, 20, and 30 min after installation of hypoosmotic perfusion conditions), dounced with an Ultraturrax (Janke & Kunkel, Staufen, Germany) at 0 °C in lysis buffer containing 20 mmol/liter Tris-HCl (pH 7.4), 140 mmol/liter NaCl, 10 mmol/liter NaF, 10 mmol/liter sodium pyrophosphate, 1% Triton X-100, 1 mmol/liter EDTA, 1 mmol/liter EGTA, 1 mmol/liter sodium vanadate, 20 mmol/liter β-glycerophosphate, and protease inhibitor mixture.

Immune Complex Kinase Assays and Western Blot Analysis—The lysed samples from the perfused liver or hepatocytes were centrifuged at 4 °C, and aliquots of the supernatant were incubated with 1.5 μg of an antibody recognizing Erk-1 and Erk-2 for 2 h at 4 °C. Immune complexes were collected by using protein A-Sepharose 4B (Sigma), washed three times with lysis buffer and four times with kinase buffer (10 mmol/liter Tris-HCl (pH 7.4), 150 mmol/liter NaCl, 10 mmol/liter $MgCl_2$, and 0.5 mmol/liter dithiothreitol), and incubated with 1 mg/ml MBP in the presence of 10 μCi [γ-^{32}P]ATP for 30 min at 37 °C. The reactions were stopped by adding 2× gel loading buffer, and activity of Erk-2 was monitored via autoradiography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% gel). To perform SDS-PAGE and Western blot analysis an identical volume of 2× gel loading buffer containing 200 mmol/liter dithiothreitol (pH 6.8) was added to the lysates. After heating to 95 °C for 5 min, the proteins were subjected to SDS-PAGE (50 μg protein/lane, 7.5% gels). Following electrophoresis, gels were equilibrated with transfer buffer (39 mmol/liter glycine, 48 mmol/liter Tris-HCl, 0.03% SDS, 20% methanol). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Amersham Biosciences). Blots were blocked overnight in 1% bovine serum albumin solubilized in 20 mmol/liter Tris-HCl, pH 7.5, containing 150 mmol/liter NaCl and 0.1% Tween 20 and then incubated for 3–4 h with antibodies raised against [$Tyr(P)^{418}$]Src, [$Tyr(P)^{529}$]Src, Src, [$Tyr(P)^{397}$]FAK, FAK, [$Thr(P)^{180}$/$Tyr(P)^{182}$]$p38^{MAPK}$ and p38 at a dilution of 1:5,000. Following washing and incubation for 2 h with horseradish peroxidase-coupled anti-rabbit-IgG antibody (1:10,000), the blots were washed again and developed using enhanced chemiluminescent detection (Amersham GmbH, Freiburg, Germany). Densitometric analysis was performed with the E. A. S. Y. RH system (Herolab, Wiesloch, Germany).

Electron Microscopy—For electron microscopic morphometry, fixation of the liver lobes was performed as described previously (11) by perfusion of glutaraldehyde (3%) in Krebs-Henseleit medium for 30 s. From the fixed livers small cubes of ~1 mm^3 were cut, postfixed for 2 h with 2% osmium tetroxide, 2% uranylacetate, and 1.5% lead citrate in PBS buffer, dehydrated in a graded series of ethanol and embedded in Epon 812. Thin sections for electron microscopy were placed on copper grids, stained with uranyl acetate and lead citrate, and were examined with a EM 900 electron microscope (Zeiss, Oberkochem, Germany).

Quantitative Evaluation of Intracellular Organelles—The autophagic vacuoles were defined as bits of cytoplasm sequestered from the remaining cytoplasm by one or two membranes. The morphology of autophagic vacuoles has been described in detail elsewhere (26). The square fields, which were defined by the copper grids (127 μm × 127 μm) were used as test fields and systematically searched for autophagic vacuoles at a magnification of ×10,500. The area of cytoplasm that was examined ranged between 7,000 and 12,000 μm^2 (n = 6). The area of the AV was measured at magnification ×21,000. Low power electron micrographs of the test fields were mounted, and the area of the hepatocytic cytoplasm was calculated by count pointing method (144 test points). The fractional volume of autophagic vacuoles, which is defined as the volume of autophagic vacuoles per volume of liver cell cytoplasm (V_{av}/V_c) was calculated as described previously (10, 27).

Immunocytochemistry and Confocal Laser Microscopy—For indirect immunofluorescence microscopy, rat livers were perfused for 120 min under isoosmotic conditions, and liver lobes were instantly fixed for cryosectioning in liquid nitrogen. When present, latrunculin B (2 μmol/liter) had been added 30 min prior to fixation of liver lobes. Liver sections were obtained using a cryotom CM 350 S (Leica, Bensheim, Germany) at a thickness of 5 μm. Air-dried samples were fixed using for 10 min at 4 °C and washed five times with ice-cold PBS. Immediately after washing samples were incubated with Phalloidin-FITC (Sigma) at a dilution of 1:500 in PBS containing 5% bovine serum albumine for 2 h at room temperature. Subsequently samples were washed again three times with ice-cold PBS. Immunostained liver perfusion samples were analyzed with a Leica TCS NT confocal laser scanning system (Leica, Bensheim, Germany) DM IRB inverted microscope. Images were acquired from a channel at a wavelength of 488 nm.

Materials—The integrin antagonistic GRGDSP and the inactive control peptide GRGESP were from Bachem (Heidelberg, Germany). The

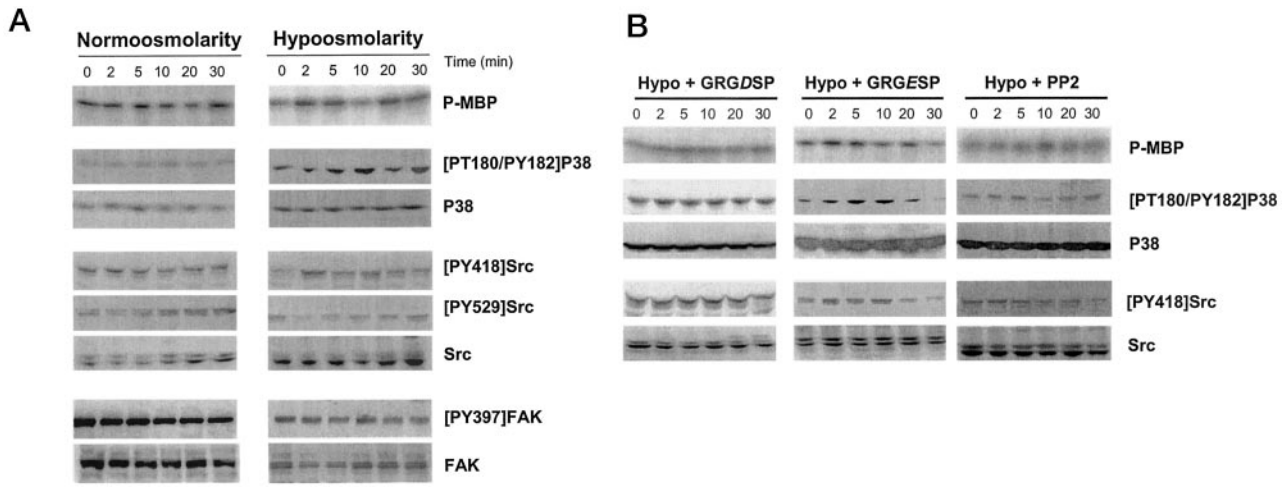

FIG. 1. **Activation of Src and MAP kinases by hypoosmotic hepatocyte swelling in perfused rat liver and its inhibition by Src inhibitor PP-2 and the integrin receptor antagonistic peptide GRGDSP, but not its inactive analogue GRGESP.** Livers from fed rats were perfused for 130 min with isoosmotic perfusion medium. Then, osmolarity was lowered from 305 to 185 mosmol/liter (*A*). Liver samples were taken immediately before (zero time point) and during perfusion with hypoosmotic medium (2, 5, 10, 20, and 30 min after onset of hypoosmotic perfusion conditions). Infusion of inhibitors was started 30 min prior to installation of hypoosmolarity (*B*). Src-Tyr^{418} and Src-Tyr^{529} phosphorylation, dual p38 phosphorylation and FAK-Tyr^{397} phosphorylation were addressed by Western blot analyses using the respective phosphospecific antibodies. In addition, expression of Src, FAK, and p38 was monitored by probing Western blots with antibodies recognizing the respective total protein. Erk-1/Erk-2 activity was measured with an immune complex assay using MBP as a substrate. Representative results of 3–5 independent experiments are shown.

TABLE I

Effects of the integrin antagonistic peptide GRGDSP, its inactive analogue GRGESP and the Src inhibitor PP-2 on hypoosmotic Src-Tyr^{418} and dual phosphorylation of p38 and Erk-1/Erk-2 activation in perfused rat liver

Data are from perfused rat liver exposed to hypoosmotic perfusion medium (185 mosmol/liter), without further pretreatment or in the presence of GRGDSP (10 μmol/liter), GRGESP (10 μmol/liter), or PP-2 (250 nmol/liter), as described in Fig. 1. Quantification was performed densitometrically. Phosphorylation of Src-Tyr^{418}, Src-Tyr^{529}, and p38-Thr^{180}/Tyr^{182} and Erk-1/Erk-2 activity under isoosmotic conditions at the zero time point was set to one, and the maximal phosphorylation/activation observed following hypoosmotic exposure is given as a fraction thereof. Changes in Src-Tyr^{418} and Src-Tyr^{529} phosphorylation and dual p38 phosphorylation were normalized to expression of the respective total protein. Data are from 3–5 separate perfusion experiments and represent means ± S.E.

Condition	Inhibitor	[Tyr(P)^{418}]Src	[Thr(P)^{180}/Tyr(P)^{182}]p38	[Tyr(P)^{529}]Src	Erk-1/Erk-2
Hypoosmotic	None	2.8 ± 0.6^a	1.9 ± 0.2^a	0.7 ± 0.1^a	2.1 ± 0.2^a
Hypoosmotic	GRGDSP	1.2 ± 0.2^b	1.1 ± 0.1^b	0.7 ± 0.2	1.1 ± 0.1^b
Hypoosmotic	GRGESP	2.1 ± 0.3^a	2.7 ± 0.8^c	0.6 ± 0.2	1.7 ± 0.1^a
Hypoosmotic	PP-2	1.1 ± 0.2^d	1.1 ± 0.2^b	0.7 ± 0.2	1.1 ± 0.1^b

^{a} Response significantly different from isoosmotic control ($p < 0.05$).
^{b} Response significantly different from the response evoked by hypoosmolarity in absence of inhibitory compounds ($p < 0.05$).
^{c} Response different from the isoosmotic control ($p = 0.06$).
^{d} Response significantly different from the response evoked by hypoosmolarity in absence of inhibitory compounds ($p = 0.07$).

antibody raised against Erk-1/Erk-2 was from Upstate (Charlottesville, VA). Antibodies recognizing [Tyr(P)^{397}]FAK, [Tyr(P)^{418}]Src, [Tyr(P)^{529}]-Src and total Src were from BIOSOURCE (Camarillo, CA). Anti-[Thr(P)^{180}/Tyr(P)^{182}]p38^{MAPK} antibody was from Promega (Madison, WI). The antibodies raised against total FAK and total p38 were from Santa Cruz Biotechnology. [γ-^{32}P]ATP, L-[4,5-^{3}H]leucine, [^{3}H]inulin, and [^{14}C]urea were from Amersham Biosciences. L-lactic acid was from Roth (Karlsruhe, Germany). Glutaraldehyde was purchased from Serva (Heidelberg, Germany). PP-2 was from Biomol (Plymouth, PA), and PP-3 and latrunculin B were from Calbiochem (Bad Soden, Germany). Dulbecco's modified Eagle's medium, fetal bovine serum, and gentamicin were purchased from Biochrom (Berlin, Germany). Fibronectin was purchased from Invitrogen (Karlsruhe, Germany). Enzymes were from Roche Applied Science. Insulin, dexamethasone, and glutamine were from Sigma. Penicillin/streptomycin was from Invitrogen. All other chemicals were from Merck (Darmstadt, Germany).

Statistics—Data from different perfusion experiments are given as means ± S.E. (number of independent experiments). Conditions were compared by Student's *t* test. Differences were considered significant at $p < 0.05$.

RESULTS

Involvement of Integrins and Src in Hypoosmotic Signal Transduction—Hypoosmotic (185 mosmol/liter) liver perfusion induced a transient Src phosphorylation on Tyr^{418}, which was maximal (2.8 ± 0.6-fold, $n = 4$) at about 2 min. The increase in Src-Tyr^{418} phosphorylation was accompanied by a transient dephosphorylation of Src on Tyr^{529}. According to earlier findings (10, 28), hypoosmolarity induced a transient activation of the MAP kinases Erk-1/Erk-2 and p38, which was maximal between 5 and 10 min (Fig. 1). Normoosmotic control perfusions were without effect on Erk-1/Erk-2 and p38^{MAPK} (10) and neither altered Src phosphorylation on Tyr^{418} and Tyr^{529}, nor FAK phosphorylation on Tyr^{397} (Fig. 1).

Infusion of the integrin antagonistic peptide GRGDSP (10 μmol/liter), but not the inactive analogue GRGESP (10 μmol/liter) prevented the hypoosmotic stimulation of Src-Tyr^{418} phosphorylation and activation of the MAP kinases Erk-1/Erk-2 and p38 (Fig. 1*B* and Table 1). Likewise, PP-2 (250 nmol/liter), an inhibitor of Src kinases (29), abolished the hypoosmotic increase of Src-Tyr^{418} phosphorylation and activation of the MAP kinases (Fig. 1*B* and Table 1). In the presence of the inhibitors no significant effect of hypoosmotic perfusion on Src-Tyr^{529} phosphorylation could be observed (Table 1).

Further experiments were performed in isolated liver cells plated on fibronectin. 48-h cultured cells were exposed to hypoosmotic (205 mosmol/liter) or normoosmotic (305 mosmol/liter) medium for 2 min. Hypoosmolarity induced an 2.7 ±

0.6-fold increase of Src-Tyr^{418} phosphorylation, which was blunted to 0.8 ± 0.1- and 1.1 ± 0.1-fold in the presence of GRGDSP (250 μmol/liter) or PP-2 (20 μmol/liter), respectively (n = 5). In a similar way, GRGDSP and PP-2 reduced the hypoosmotic p38 activation from 2.3 ± 0.4-fold under hypoosmotic control conditions to 0.9 ± 0.1- and 1.3 ± 0.2-fold (n = 5) in the presence of the respective inhibitors (Fig. 2). The findings support the suggestion that direct rather than indirect effects of GRGDSP and PP-2 account for the inhibitory effects found in the intact liver.

The Integrin/Src System Is Involved in Regulatory Volume Decrease—As shown recently, p38^{MAPK} activation in response to hypoosmotic cell swelling is also involved in regulatory volume decrease (RVD) (8), which manifests within about 10 min of hypoosmotic exposure as a net K^+ and Cl^- release from the hepatocytes through Ba^{2+}-, DIDS-, and quinidine-sensitive ion channels (30). This RVD response only partially restores cell volume, and after its completion the cells are left in a slightly swollen state (30). When perfused livers are suddenly exposed to hypoosmotic fluid (225 mosmol/liter), a net K^+ release of 12.2 + 0.5 μmol/g of liver is observed, which is completed within 415 ± 11 s (Table 3), and the residual cell volume increase after completion of RVD is 13.4 + 0.8% (Table 2). As shown recently (8), inhibition of p38^{MAPK} blunts and delays this volume regulatory net K^+ release and renders the cells in a more swollen state. As shown in Table 2, prevention of swelling-induced p38^{MAPK} activation by GRGDSP or PP-2 rendered the cells in a significantly more swollen state following hypoosmotic exposure. Likewise, volume regulatory net K^+ efflux was significantly decreased and delayed in the presence of GRGDSP and PP-2, compared with the presence of their inactive analogues GRGESP and PP-3, respectively (Table 3). These data suggest that the integrin/Src system is also involved in triggering RVD via p38^{MAPK} activation.

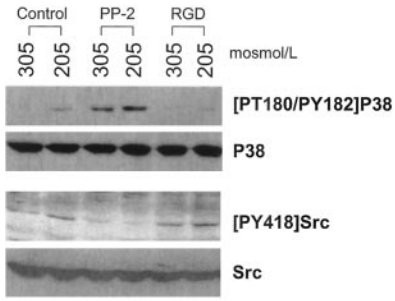

FIG. 2. **Sensitivity of hypoosmotic activation of Src and p38^{MAPK} toward inhibition by GRGDSP and PP-2 in cultured rat hepatocytes.** Liver parenchymal cells were isolated from livers of male Wistar rats by collagenase perfusion. After a total cultivation time of 48 h, cells were starved for 4 h. After this time, cells were either exposed to hypoosmolar (205 mosmol/liter) or normoosmotic control medium (305 mosmol/liter) for 2 min. If present, inhibitors (GRGDSP (RGD) or PP-2) had been added 4 h prior to installation of hypoosmotic conditions. At the end of this treatment, medium was removed from the culture, cells were immediately lysed and phosphorylation of Src-Tyr^{418}, and dual phosphorylation of p38 was addressed by Western blot analysis as described in the legend to Fig. 1. Results were normalized to total blotted protein, as measured by determination of total p38 and Src. Representative results of 4–5 independent experiments are shown.

Role of the Integrin/Src System in Proteolysis Control by Cell Volume—As shown previously (10, 11, 31, 32), hypoosmotic cell swelling led to a rapid and transient inhibition of proteolysis in perfused rat liver (Fig. 3) due to an inhibition of autophagic vacuole formation. Upon normoosmotic reexposure, proteolysis rate returned to baseline levels, indicating the reversibility of the process. GRGDSP, a hexapeptide with integrin receptor antagonistic activity (33), but not its inactive analogue GRGESP, prevented the swelling-dependent decrease of autophagic proteolysis in perfused rat liver (Fig. 3*A*). In the presence of GRGESP, the decrease of proteolysis upon hyposmolar exposure (225 mosmol/liter) was 23.1 ± 3.4% (n = 6), whereas GRGDSP reduced this effect by about 80% to 5.6 ± 2.1% (n = 4). Also Src inhibition by PP-2 abolished the antiproteolytic effect of hypoosmotic cell swelling. In the presence of PP-2, hypoosmotic proteolysis inhibition was only 3.1 ± 1.3% (n = 4) compared with an inhibition by 21.9 ± 2.9% (n = 3) in the presence of PP-3, a biologically inactive analogue of PP-2 (Fig. 3*B*). Likewise, in the presence of a higher degree of hypoosmolarity (185 mosmol/liter), proteolysis inhibition was 33.2 ± 1.4% under control conditions (n = 6) and was blunted to 9.6 ± 2.9% (n = 4) in the presence of GRGDSP (10 μmol/liter), an inhibitory effect, which was not observed with GRGESP (hypoosmotic proteolysis inhibition 31.0 ± 3.4% (n = 3)). Neither PP-3 nor GRGESP had any significant influence on swelling-induced proteolysis inhibition.

Also under conditions of isoosmotic hepatocyte swelling, *i.e.* by addition of amino acids, an involvement of integrin-dependent Src activation in triggering the inhibition of autophagic proteolysis could be demonstrated. Glutamine is known to exert its antiproteolytic action mainly via an increase in cell hydration ((23, 31, 34), compare also Table 2). Whereas glutamine inhibited proteolysis by 14.0 ± 1.2% (n = 6) under control conditions (Fig. 4*A*), this effect was decreased to 3.9 ± 1.0% (n = 4) in the presence of the integrin antagonistic peptide

TABLE II

Cell hydration changes in perfused rat liver under the influence of anisoosmotic conditions and amino acids in presence or absence of the integrin inhibitory peptide GRGDSP, its inactive analogue GRGESP, and the Src inhibitor PP-2

Livers from male, fed Wistar rats were perfused for 160 min. Hypotonic perfusion conditions or amino acid infusions were installed from 130 to 160 min. If present, inhibitors were infused 30 min prior to installation of hypotonic perfusion conditions or infusion of glutamine, glutamine plus glycine or phenylalanine. Intracellular water space under control conditions was 551 ± 10 μl/g liver weight (n = 28). Data refer to hydration changes 30 min after institution of the respective challenges, i.e. after completion of volume regulatory ion fluxes. No detectable changes of cell hydration were observed with GRGDSP (10 μmol/liter), GRGESP (10 μmol/liter) or PP-2 (250 nmol/liter). For experimental details, see "Experimental Procedures." In parentheses, the number of different perfusion experiments is given. Values are given as means ± S.E. from independent perfusion experiments.

Condition	Change of intracellular water space			
	Control	GRGDSP	GRGESP	PP-2
	% change of basal value			
Hypotonic (−80 mosmol/liter)	+13.4 ± 0.8 (11)	+16.9 ± 0.6 (8)[a]	+12.9 ± 2.0 (3)	+17.3 ± 0.7 (3)[b]
Hypotonic (−120 mosmol/liter)	+19.5 ± 1.3 (13)	+24.5 ± 0.9 (4)[b]	+20.0 ± 2,7 (3)	N.D.[c]
Glutamine (2 mmol/liter)	+6.4 ± 0.6 (3)	+7.9 ± 1.5 (3)	N.D.	+8.3 ± 0.5 (3)
Glutamine + glycine (2 mmol/liter)[d]	+22.9 ± 1.9 (4)	+24.0 ± 3.0 (3)	+21.9 ± 2.9 (4)	N.D.
Phenylalanine (2 mmol/liter)	+0.8 ± 0.3 (5)	+1.0 ± 0.8 (3)	N.D.	+1.2 ± 1.0 (3)

[a] Significant difference from the respective control in the absence of inhibitors, $p < 0.05$.
[b] $p < 0.01$.
[c] ND, not determined.
[d] Livers from 24-h starved rats were used.

TABLE III

Influence of the integrin antagonistic peptide GRGDSP, the Src inhibitor PP-2, and p38^{MAPK} inhibitor SB 203 580 on the extent and time course of cell volume regulatory net K^+ release in response to hypoosmotic exposure (225 mosmol/liter) of isolated perfused rat liver

Livers from male, fed Wistar rats were exposed to hypoosmotic perfusion medium (305 → 225 mosmol/liter) for 30 min. Inhibitors were dissolved in Me_2SO and were infused continously, starting 30 min prior to installation of hypoosmotic perfusion conditions. Calculations of net K^+ fluxes and of duration of K^+ release were made by planimetry as described earlier (8). In control experiments, Me_2SO (20 μl/min) was present. N denotes the number of different perfusion experiments. Symbols, significant difference from control.

Condition	Net K^+ release	K^+ release time	N
	μmol/g	*s*	
Control	12.2 ± 0.5	415 ± 11	5
GRGDSP (10 μmol/liter)	7.6 ± 0.4[a]	530 ± 40[b]	8
GRGESP (10 μmol/liter)	10.2 ± 0.4[c]	395 ± 19[c]	5
PP-2 (250 nmol/liter)	8.3 ± 0.2[d]	651 ± 43[c]	5
PP-3 (250 nmol/liter)	11.7 ± 0.4[c]	458 ± 18[c]	6
SB 203580 (250 nmol/liter)	6.7 ± 0.3[a]	597 ± 38[a]	6

[a] $p < 0.001$.
[b] $p < 0.05$.
[c] Not significant.
[d] $p < 0.01$.

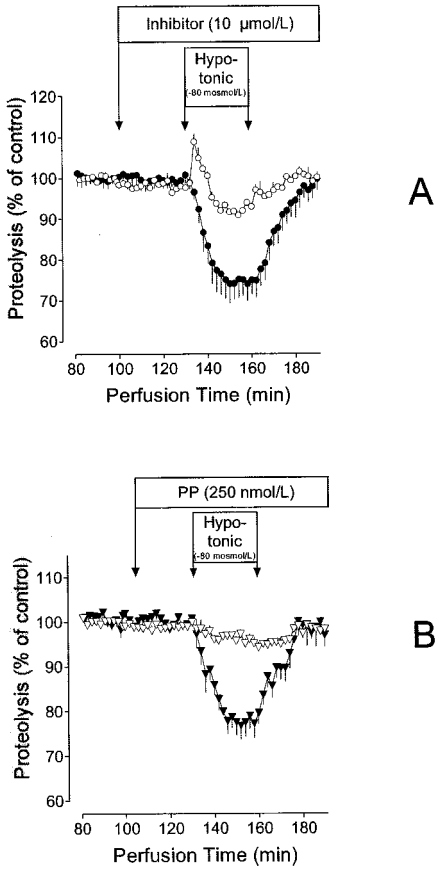

FIG. 3. **Antiproteolytic effect of hypoosmolarity and its sensitivity to the integrin receptor antagonist GRGDSP (*A*) and Src inhibitor PP-2 (*B*) in perfused rat liver.** Livers from fed rats were prelabeled *in vivo* by intraperitoneal injection of 150 μCi of [^{3}H]leucine, and ^{3}H label release into effluent was monitored as a measure of hepatic proteolysis in liver perfusion experiments. Due to different labeling of the animals *in vivo*, the release of radioactivity was set to 100% during control conditions. Infusion of integrin antagonistic peptide GRGDSP (Ref. 33, ○) or its inactive analogue GRGESP (●) was started at 100 min of perfusion time (*A*). In *B*, either PP-2 (250 nmol/liter, ▽), an inhibitor of Src activation (29) or its inactive analogue PP-3 (250 nmol/liter, (▼) (50)) was infused 30 min prior to installation of hypoosmotic perfusion conditions. Neither GRGDSP, GRGESP, PP-2, nor PP-3 had a significant effect on basal proteolysis activity. Data are given as means ± S.E. and are from 4–5 separate perfusion experiments, respectively.

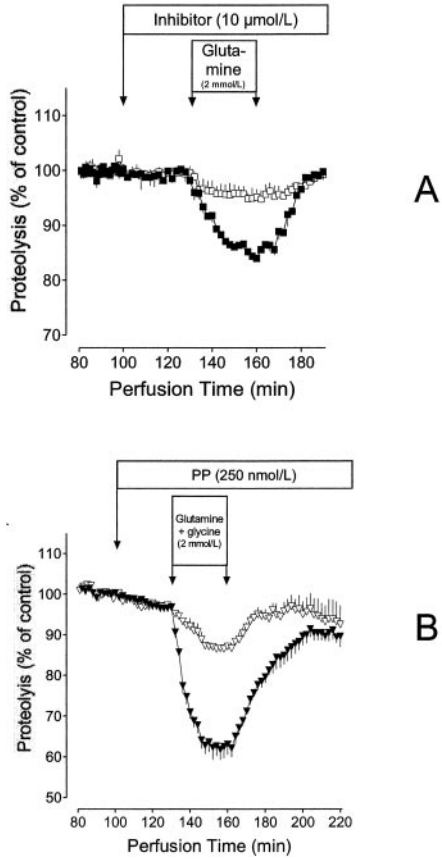

FIG. 4. **Antiproteolytic effect of glutamine and glycine and its abolition by GRGDSP and PP-2 in perfused rat liver.** Conditions were as shown in Fig. 3. Livers from fed rats were used in *A*. In the presence of either GRGDSP (10 μmol/liter, □) or GRGESP (10 μmol/liter, ■), glutamine (2 mmol/liter) was infused for 30 min (*A*). Livers from 24-h starved rats were used in *B*. In the presence of either PP-2 (250 nmol/liter, ▽) or PP-3 (250 nmol/liter, ▼), glutamine plus glycine (2 mmol/liter, each) were infused for 30 min. Inhibitors were installed 30 min prior to infusion of amino acids. Results are from 3–4 experiments for each condition, data are shown as means ± S.E.

GRGDSP (10 μmol/liter). Likewise, in livers from 24-h starved animals, in which the swelling potency of amino acids is increased due to an up-regulation of concentrative amino acid transport systems in the plasma membrane (35, 36), the strong inhibition of proteolysis by glutamine+ glycine (2 mmol/liter, each) by 34.6 ± 0.8% ($n = 4$) was diminished to 9.8 ± 1.0% ($n = 3$) in the presence of PP-2 (250 nmol/liter, Fig. 4*B*). This residual antiproteolytic activity of these amino acids in the presence of PP-2 resembles that obtained after p38^{MAPK} inhibition (10) and is ascribed to ammonia formation during breakdown of these amino acids with consecutive alkalinization of the degradative compartments (34). In contrast to glutamine and glycine, phenylalanine does not induce hepatocyte swelling and its antiproteolytic action is p38^{MAPK}-independent and involves mechanisms distinct from hepatocyte swelling (10, 11, 32). As shown in Fig. 5, there was no effect of GRGDSP on the antiproteolytic effect of phenylalanine. These data suggest that dependence on the integrin/Src system is a feature of proteolysis control by cell volume, but not of proteolysis control in general, *i.e.* also by cell volume-independent mechanisms.

Integrins are linked to the actin cytoskeleton (17). Thus, the role of microfilaments in hypoosmotic signaling toward proteolysis inhibition was assessed after destruction of microfilaments by latrunculin B. Latrunculin B (2 μmol/liter) induced sustained cholestasis in perfused rat liver (Fig. 6*A*), and as

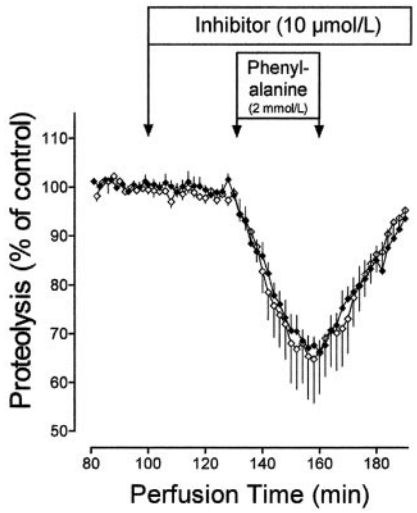

FIG. 5. **Lack of effect of integrin antagonistic peptide GRGDSP on phenylalanine-dependent decrease of proteolysis in perfused rat liver.** Conditions were as described in the legend to Fig. 3. Livers from fed rats were perfused with isoosmotic perfusion medium. Phenylalanine (2 mmol/liter) was present from 130–160 min. Either GRGDSP (10 μmol/liter, ◇) or GRGESP (10 μmol/liter, ◆) were present from the 100-min perfusion time. Results are from three experiments for each condition. Data are shown as means ± S.E.

shown in Fig. 7, microscopically polymerized actin was no longer visible in immunofluorescence-labeled thin sections of perfused rat liver. However, despite destruction of actin filaments, in the presence of latrunculin the antiproteolytic effect of hypoosmotic cell swelling was fully preserved (Fig. 6*B*). These findings indicate that integrin-dependent proteolysis regulation by cell swelling does not require the integrity of microfilaments.

Sequestration of Autophagic Vacuoles—Morphometric analysis of electron micrographs (Table 4) from perfused rat liver was performed under hypoosmotic conditions and in the presence of phenylalanine, a non-swelling amino acid with strong antiproteolytic activity. Shifting ambient osmolarity from 305 (control) to 185 mosmol/liter (hypoosmotic) significantly decreased the fractional volume occupied by autophagic vacuoles (V_{av}/V_c) within 30 min from $51.8 \pm 1.9 \times 10^{-4}$ ($n = 7$) by about 47% to $27.3 + 3.0 \times 10^{-4}$ ($n = 6$), indicating that hypoosmotic proteolysis inhibition is due to an inhibition of autophagic vacuole formation (10). The hypoosmolarity induced decrease of V_{av}/V_c was fully suppressed by PP-2 (250 nmol/liter) and significantly inhibited by GRGDSP (10 μmol/liter), but not GRGESP (10 μmol/liter, Table 4). In line with the known strong antiproteolytic effect of phenylalanine (37), which neither causes significant ammonia production nor changes of liver cell hydration (10, 11, 32), phenylalanine also caused a marked decrease of V_{av}/V_c (Table 4). The phenylalanine-dependent decrease of autophagic vacuole formation, however, was insensitive to inhibition by PP-2 (Table 4). In view of the fact that the swelling-independent mechanisms of regulation of protein degradation do not require intact microtubules (32) and activation of $p38^{MAPK}$ (11) the data suggest that the swelling-dependent (hypoosmolarity, glutamine, glycine) and swelling-independent proteolysis regulation mechanisms (phenylalanine) converge at the level of formation of autophagic vacuoles (sequestration step), with the former being mediated by integrin-triggered Src-dependent $p38^{MAPK}$ activation, and the latter being integrin/Src-and $p38^{MAPK}$-independent.

DISCUSSION

Cell hydration changes critically determine cell function by initiating signal transduction (osmosignaling). Here evidence

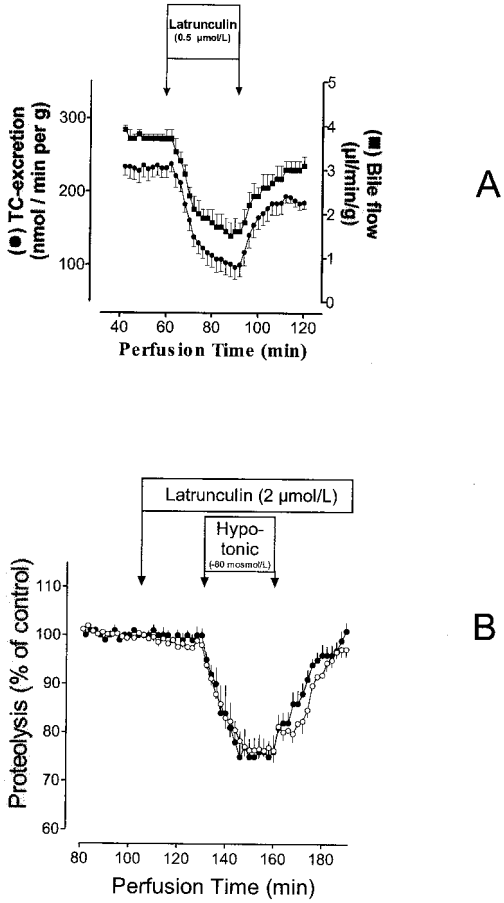

FIG. 6. **Effect of latrunculin B on taurocholate excretion and bile flow under isoosmotic conditions (*A*) and proteolysis inhibition by hypoosmolarity (*B*).** In perfused rat liver, bile flow (■) and taurocholate excretion into bile (●) in the presence of latrunculin B (0.5 μmol/liter) were assessed by gravimetry and established tracer techniques (*A*). Results are from three independent experiments. In livers from rats that had been prelabeled *in vivo* with [^{3}H]leucine, release of ^{3}H label was followed as an indicator of hepatic proteolysis (*B*). Latrunculin B (2 μmol/liter) was infused for 30 min prior to installation of hypoosmolar perfusion conditions (225 mosmol/liter, ○). In control experiments (●), hypoosmotic conditions were installed in absence of the microfilament inhibitor. Results are from four independent experiments each, and values reflect means ± S.E.

is presented that integrins play a role in sensing hepatocyte swelling induced by hypoosmolarity or amino acid accumulation in perfused rat liver, *i.e.* an intact organ model authentically preserving hepatocyte polarity and three-dimensional anchoring to the extracellular matrix. Based on experiments with the fibronectin-derived hexapeptide GRGDSP (33) and the Src inhibitor PP-2 (29), this study shows a role of integrin-mediated activation of Src-type kinases as a trigger of $p38^{MAPK}$ and Erk-1/Erk-2 activation by hepatocyte swelling (Figs. 1 and 2). In line with this, the swelling-related reduction of autophagosome volume (Table 4), proteolysis inhibition (Figs. 3 and 4) and the RVD and cell swelling induced by hypoosmolarity (Tables 2 and 3), which represent functional consequences of $p38^{MAPK}$ activation, are blunted by the RGD peptide and PP-2, respectively. Proteolysis inhibition by phenylalanine, which does not involve cell swelling and $p38^{MAPK}$ (10) is insensitive to the RGD peptide and PP-2 (Fig. 5), indicating that the inhibitors do not generally interfere with the regulation of autophagic proteolysis and specifically impair the swelling-related signal transduction toward proteolysis.

In contrast to results from the isolated perfused rat liver (23) and 24-h cultured rat hepatocytes (32), no antiproteolytic re-

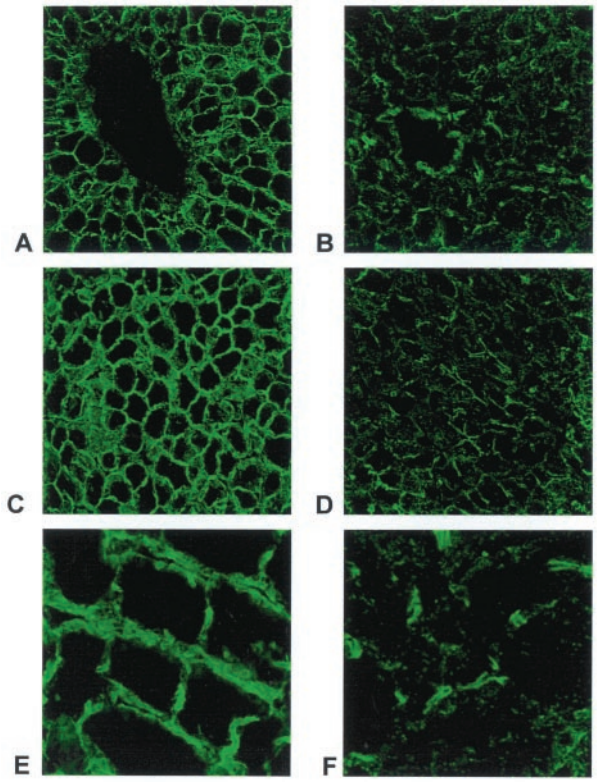

FIG. 7. **The effect of latrunculin B (2 μmol/liter) on microfilaments in perfused rat liver.** Morphological analysis of the distribution of actin G was obtained in slices from perfused rat liver by freeze sectioning under control conditions after a 90-min isoosmotic perfusion period without inhibitor (*A*, *C*, *E*) or after prior infusion of latrunculin B (2 μmol/liter) from 60 to 90 min perfusion time (*B*, *D*, *F*). Microfilaments were visualized by means of immunofluorescence labeling with phalloidin-targeted mouse anti-actin-G antibodies. Representative results are shown from three independent perfusion experiments for each condition and show native transmission microscopic views (*A* and *B*, magnification ×43), projection scans (*C* and *D*, magnification ×43), and enlarged pericellular areas (*E* and *F*, magnification ×128). For further details, see "Experimental Procedures."

sponse to hypoosmolarity was found in freshly isolated suspended rat hepatocytes (38). Likewise, hypoosmolarity activates Erk-1/Erk-2 in perfused rat liver (Refs. 8 and 10 and this study) and cultured hepatocytes (39), but not in freshly isolated cells (40). In view of the present study, the absence of MAP kinase activation and proteolysis regulation by hypoosmolarity in suspended, but not in 48-h cultured hepatocytes (Fig. 2) may reflect a deficit in sensing hypoosmotic swelling due to the lack of integrin-mediated adherence to the extracellular matrix. On the other hand, in freshly isolated suspended hepatocytes hypoosmolarity activates PI 3-kinase leading to increased glycogen and fatty acid synthesis (40, 41) and taurocholate uptake (42). Further, hypoosmolarity sensitizes these cells to proteolyis inhibition by amino acids (38), which depends on ribosomal S6 phosphorylation in a rapamycin-sensitive manner (13). This suggests that multiple osmosensing mechanism exist in hepatocytes, which could be differentially linked to intracellular signaling pathways.

Although hypoosmotic swelling was shown to induce reorganization of the actin cytoskeleton in isolated hepatocytes (43) and FAK tyrosine phosphorylation in HepG2 hepatoma (44) and intestine 407 cells (45) and FAK is involved in mechanosensing in fibroblasts (46), it seems questionable that actin filaments and FAK play a role in integrin-mediated signaling toward swelling-induced proteolyis inhibition in perfused rat liver. Disruption of the actin cytoskeleton by cytochalasins prevents integrin signaling toward FAK and Erk-1/Erk-2 in

TABLE IV

Effects of hypoosmotic exposure and phenylalanine on fractional volume (V_{av}/V_c) of autophagic vacuoles (AVs) in the perfused rat liver in presence of the integrin antagonistic peptide GRGDSP, its inactive analogue GRGESP, the Src inhibitor PP-2, or the $p38^{MAPK}$ inhibitor SB 203580

Livers from fed male Wistar rats were perfused in an open non-recirculating manner for a total time of 160 min. Hypotonic perfusion medium (185 mosmol/liter) or phenylalanine infusion (2 mmol/liter) was installed from 130 to 160 min. At 160 min the livers were perfused with 3% glutaraldehyde for 30 s and tissues were further processed for transmission electron microscopy. When present, inhibitors, i.e. GRGDSP (10 μmol/liter), its inactive analogue GRGESP (10 μmol/liter), Src inhibitor PP-2 (250 nmol/liter) or $p38^{MAPK}$ inhibitor SB 203580 (250 nmol/liter) were started 30 min prior to hypoosmolarity or amino acid infusion and infused from 100 to 160 min. Results are expressed as fractional autophagic vacuole volume, which is defined as the volume of AVs per cytoplasmic volume (V_{av}/V_c). Data are given as means ± S.E. N, the number of experiments. Significance was calculated by means of Student's *t* test. Symbols are significance *versus* respective isoosmotic control in the absence of amino acids.

Condition	Inhibitor	$V_{av}/V_c \times 10^{-4}$	N
Control	Absent	51.8 ± 1.9	7
Hypoosmotic	Absent	27.3 ± 3.0[a]	6
	GRGDSP	43.4 ± 4.1[b]	4
	GRGESP	24.3 ± 2.3[a]	3
	PP-2	51.4 ± 8.0[b]	3
	SB 203 580[c]	53.0 ± 6,9[b]	3
Phenylalanine	Absent	28.4 ± 3.9[a]	3
	PP-2	21.7 ± 3.3[a]	4

[a] $p < 0.001$.
[b] Not significant.
[c] Data from Ref. 10.

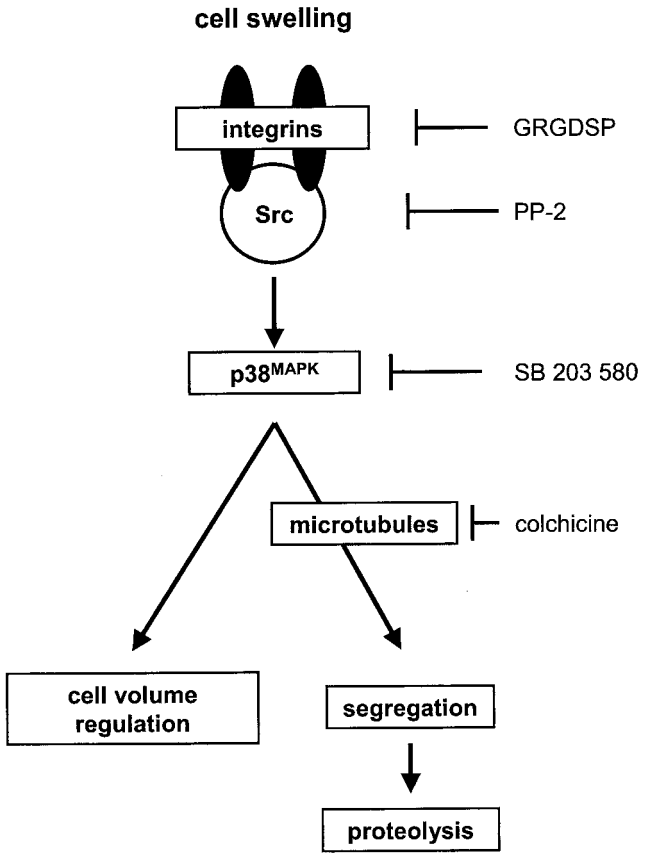

FIG. 8. **Hypothetical scheme of proteolysis regulation by cell volume in liver.**

NIH-3T3 cells (47), abolishes hypoosmotic FAK Tyr phosphorylation in HepG2 cells (44) and produces a pronounced cholestasis in perfused rat liver (32). However, cytochalasin treat-

ment does not interfere with the antiproteolytic response to hypoosmolarity in the latter system (32). Likewise, latrunculin B, which disturbs actin organization by a mechanism distinct from that of cytochalasins, induces cholestasis and disrupts the actin cytoskeleton in perfused rat liver but does not impair hypoosmotic proteolysis inhibition (Figs. 6 and 7). Secondly, [Tyr(P)^{397}]FAK phosphorylation, which is present already under normoosmotic conditions in perfused rat liver, does not increase in response to hypoosmolarity (Fig. 1). Finally, livers are perfused in absence of serum, a condition known to prevent Src recruitment by FAK (17). FAK-dependent and independent pathways of Src activation are triggered by integrins (17) and such independent pathways are apparently involved in swelling-induced MAP kinase signaling toward proteolysis inhibition under the conditions employed in the present study. Potential integrin partners in osmosensing and signaling include tetraspan proteins such as the osmotically regulated CD9 (48) and caveolin, which mediates the hypoosmotic activation of volume regulatory anion channels in endothelial cells (49). A link of integrins to the microtubular system may also play a role in generating antiproteolytic signals apart from/downstream of hypoosmotic p38^{MAPK} activation.

Our current working hypothesis is outlined in Fig. 8. Integrins sense hepatocyte swelling, leading to activation of Src-type kinases, which in turn mediate activation of Erk-1/Erk-2 and p38^{MAPK}. Impairment of integrin-matrix interaction and inhibition of Src-type kinases, but not disruption of the actin cytoskeleton prevents the p38^{MAPK}-dependent inhibition of autophagy due to cell swelling and the regulatory volume decrease triggered by hypoosmolarity. Thus, integrins may act as cell volume sensors at least in response to hepatocyte swelling. As in bacteria, plants, and fungi (16), multiple osmosensing mechanisms probably also exist in mammalian cells, and future work will reveal their relative contributions.

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