Activation of extracellular signal-regulated kinases Erk-1 and Erk-2 by cell swelling in H4IIE hepatoma cells

Freimut SCHLIESS, Rainer SCHREIBER and Dieter HÄUSSINGER*
Medizinische Klinik der Heinrich Heine-Universität Moorenstraße 5, D-40225 Düsseldorf, Germany

Hepatic metabolism and gene expression are among the factors controlled by the cellular hydration state, which changes within minutes in response to aniso-osmotic environments, cumulative substrate uptake, oxidative stress and under the influence of hormones such as insulin. The signalling events coupling cell-volume changes to altered cell function were studied in H4IIE rat hepatoma cells. Hypo-osmotic cell swelling resulted within 1 min in a tyrosine kinase-mediated activation of the extracellular signal-regulated protein kinases Erk-1 and Erk-2, which was independent of protein kinase C and cytosolic calcium. Activation of mitogen-activated protein kinases was followed by an increased phosphorylation of c-Jun, which may explain our recently reported finding of an about 5-fold increase in *c-jun* mRNA level in response to cell swelling. Pretreatment of cells with pertussis or cholera toxin abolished the swelling-induced activation of Erk-1 and Erk-2, suggesting the involvement of G-proteins. Thus, a signal-transduction pathway resembling growth factor signalling is activated already by osmotic water shifts across the plasma membrane, thereby providing a new perspective for adaption of cell function to alterations of the environment.

INTRODUCTION

It has recently become evident, that alterations in hepatocellular hydration, which reflect changes in liver cell volume on a short-term time-scale, play an important role in the control of hepatic metabolism and gene expression [1,2]. Such alterations in cell volume occur within minutes under the influence of hormones [1,3], oxidative stress [4,5] and cumulative amino acid uptake [6]. Cell swelling acts like an anabolic signal: it stimulates protein and glycogen synthesis and simultaneously inhibits glycogenolysis and proteolysis [1,7]. Moreover, the expression level of the phosphoenolpyruvate carboxykinase, is diminished during hypo-osmotic exposure of perfused rat liver and H4IIE rat hepatoma cells, but is elevated under hyperosmotic conditions [8]. Other examples of hydration-regulated gene expression are the ornithine decarboxylase [9], the transcription factor c-Jun, whose mRNA levels increase during cell swelling [10] and viral replication [11]. Cell swelling also leads to a rapid alkalinization of endocytotic vesicles [12,13] with potential implications for receptor–ligand sorting. The intracellular signalling events which link cell function to changes of liver cell hydration are still unknown, although some cell-volume dependent pathways such as proteolysis, bile acid transport and vesicular alkalinization have been shown to require intact microtubular structures. The aim of this study was to identify the signalling events which occur in response to cell swelling. Extracellular signal-regulated protein kinases Erk-1 and Erk-2, which belong to the family of mitogen-activated protein (MAP) kinases, are key elements in growth factor signalling [14]. They phosphorylate multiple protein substrates like the microtubule-associated proteins MAP-2 and Tau, other protein kinases like S6 kinase and transcription factors such as c-Jun [15]. MAP kinases are activated by phosphorylation at neighbouring threonine and tyrosine residues by MAP kinase kinase (MEK), which leads to a characteristic retardation of the mobility of the Erk proteins during SDS gel electrophoresis, detectable on Western blots [16].

Here we report on the activation of Erk-1 and Erk-2 in response to cell swelling, which is followed by a transient increase in c-Jun phosphorylation. This activation is sensitive to the G-protein modulators pertussis and cholera toxin, the tyrosine kinase inhibitor genistein but not to the protein kinase C (PKC) inhibitor Gö6850. Intracellular calcium shifts are not involved in upstream events, leading to the swelling-induced activation of MAP kinases.

MATERIAL AND METHODS

Cell culture and hypo-osmotic stimulation

H4IIE-C3 rat hepatoma cells (ATCC CRL 1600) were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12/ 5% CO_2/5 mM glucose at 37 °C, pH 7.4, supplemented with 10% fetal calf serum (FCS). When cells had reached 90% confluency, they were washed with Dulbecco's PBS and the culture was continued in serum-free medium for an additional 24 h. Following 5 min preincubation with 500 μM sodium vanadate, hypo-osmolarity (205 mosmol/l) was achieved by diluting the normo-osmolar medium with the appropriate volume of NaCl-free medium, but containing 500 μM sodium vanadate. In the normo-osmotic control (305 mosmol/l), the same volume of normo-osmotic medium, containing 500 μM vanadate, was added. Normo-osmotic raffinose medium was generated by substitution of 50 mM sodium chloride for 100 mM raffinose.

Abbreviations used: Erk, extracellular signal-regulated protein kinase; MAP kinase, mitogen-activated or microtubule-associated protein kinase; MEK, MAP kinase kinase; PKC, protein kinase C, PMA, phorbol 12-myristate 13-acetate; HOG1, high-osmolarity glycerol response 1; MBP, myelin basic protein; PMSF, phenylmethanesulphonyl fluoride; NP40, Nonidet P.40; DTT, dithiothreitol; FCS, fetal calf serum; KHB, Krebs-Henseleit medium; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

* To whom correspondence should be addressed.

Preparation of cell lysates

After exposure to hypo-osmotic and normo-osmotic medium respectively, medium was removed and cells were immediately, without washing procedures, lysed at 4 °C using 50 mM Tris/HCl, pH 7.2/150 mM NaCl/40 mM NaF/5 mM EDTA/5 mM EGTA/1 mM vanadate/0.5 mM phenylmethanesulphonyl fluoride (PMSF)/0.1% aprotinine/1% Nonidet P.40 (NP40)/0.1% sodium desoxycholate/0.1% SDS. The collected cells were homogenized and centrifuged at 20000 *g* and 4 °C. The supernatant was subjected to electrophoretic analysis of phosphorylation.

Kinase shift assay

One volume of gel loading buffer [pH 6.8, containing 200 mM dithiothreitol (DTT)] was added to the supernatant. After heating to 95 °C for 5 min the proteins were subjected to SDS gel electrophoresis (50 μg of protein per lane, 9% gel). Following electrophoresis, gels were equilibrated with transfer buffer (39 mM glycine/48 mM Tris-base/0.03% SDS/20% (v/v) methanol). Proteins were transferred to nitrocellulose membranes, using a semi-dry transfer apparatus (Pharmacia) according to the manufacturer's recommendations. Blots were blocked in 5% BSA-containing 20 mM Tris, pH 7.5/150 mM NaCl/0.1% Tween 20 (TBST), then probed with antisera (dilution 1:50000) at 4 °C overnight. Following washing with TBST and incubating with horseradish peroxidase-coupled anti-(rabbit IgG) antibody (dilution 1:10000) at room temperature for 1 h, the blot was washed extensively and developed using enhanced chemiluminescent detection (Amersham). Antibodies were purchased from UBI (anti-Erk-1, anti-Erk-2) and Santa Cruz Biotechnology (anti-c-Jun, anti-Raf-1).

Immune-complex kinase assay

The assay was performed as described [17]. Briefly, aliquots of cell lysate [lysis buffer: 1% Triton X-100/150 mM NaCl/10 mM Tris, pH 7.4/1 mM EDTA/1 mM EGTA/0.2 mM vanadate/0.2 mM phenylmethanesulphonyl fluoride (PMSF)/0.5% NP 40] were incubated with 2.5 μg of a monoclonal antibody raised against Erk-2 (UBI, class IgG2a) for 2 h. Immune complexes were collected by using protein A–Sepharose 4B (Pharmacia), washed three times with the lysis buffer and six times with kinase buffer (10 mM Tris/(HCl) pH 7.4/150 mM NaCl/10 mM $MgCl_2$/0.5 mM DTT) and incubated with 0.5 mg/ml myelin basic protein (MBP), 25 μM ATP and 5 μCi [^{32}P]ATP for 30 min at 37 °C. The activity state of Erk-2 was monitored via autoradiography after SDS/PAGE (12.5% gel).

Calcium imaging on the single-cell level

H4IIE cells were grown on coverslips in DMEM-F12+10% FCS. Sub-confluent cells were incubated with Krebs–Henseleit medium (KHB: 115 mM NaCl/25 mM $NaHCO_3$/5.9 mM KCl/1.18 mM $MgCl_2$/1.23 mM NaH_2PO_4/1.2 mM Na_2SO_4/1.25 mM $CaCl_2$), containing 5 μM of the fluorescent calcium chelator fura-2 acetomethoxyester and 0.02% pluronic F-127, for 30 min at 37 °C and 5% CO_2. For fluorescence recording, the coverslips were continuously superfused at a rate of 15 ml/min with KHB at 37 °C, equilibrated with O_2/CO_2 (95:5; v/v), resulting in pH 7.4. Measurement of intracellular calcium was performed as described [18].

RESULTS

Activation of Erk-1 and Erk-2 in response to hypo-osmotic cell swelling

Alterations in the phosphorylation of endogenous Erk-1 and Erk-2 of the rat hepatoma cell line H4IIE-C3 in response to cell swelling were detected using the band shift assay. Figure 1 shows that sudden reduction of the medium osmolarity from 305 to 205 mosmol/l by lowering the NaCl concentration, induced a shifting of Erk-1 and Erk-2 toward higher molecular masses within 1 min, which became markedly pronounced after 10 min. Exposure to normo-osmotic control medium was ineffective. In order to amplify the swelling-induced phosphorylation signal, the cells were preincubated with 500 μM sodium vanadate, which was also present during hypo-osmolar stimulation and in the normo-osmotic control. Under normo-osmotic conditions no increase in shifted Erk-forms was seen, even if the exposure period was extended up to 1 h (Figure 1). The correspondence between the mobility-shifts and kinase activity to MBP was demonstrated for Erk-2 (Figure 1) and Erk-1 (results not shown). It is concluded that the observed Erk-activation in H4IIE cells results from the hypo-osmotic stimulus and is amplified in the presence of vanadate. In the absence of vanadate only weak phosphorylation signals which were inconsistent and difficult to pick up, were found. Erk phosphorylation was not due to a decreased Na^+ or Cl^- activity in the medium: no shifts were observed when 50 mM NaCl in the medium was replaced by 100 mM raffinose, thereby maintaining normo-osmotic conditions (results not shown).

Both hypo-osmolarity and vanadate do not affect intracellular calcium in H4IIE cells

We evaluated whether the exposure of H4IIE cells to vanadate or hypo-osmolarity and vanadate caused changes in cytosolic free calcium. H4IIE cells were loaded with the fluorescence indicator fura-2 and the fluorescence ratio 340/380 nm was measured at the single-cell level. Neither vanadate, nor vanadate and hypo-osmolarity but ATP as a positive control, elicited a calcium signal (Figure 2). The basal intracellular free calcium level ($[Ca^{2+}]_i$) was 194±16 nM (n = 27). Thus, the hypo-osmolarity-induced Erk activation in the presence of vanadate does not result from calcium signalling events.

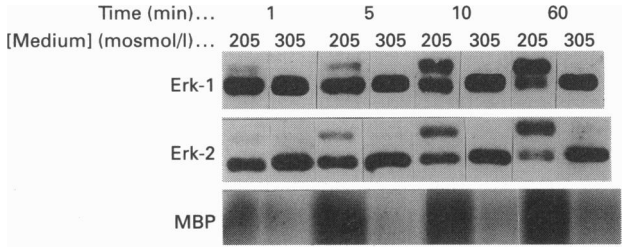

Figure 1 Activation of Erk-1 and Erk-2 in response to cell swelling in H4IIE cells, determined by mobility shifts and immune-complex assay

Following 5 min preincubation with 500 μM vanadate, cells were treated with hypo-osmotic medium (205 mosmol/l) or normo-osmotic control-medium (305 mosmol/l) respectively, for the times indicated, while maintaining the vanadate concentration constant. Representative results of four independent experiments are shown. The extract of total protein was analysed by Western blot, using antibodies specifically raised against Erk-1 and Erk-2 respectively. The immune-complex assay was performed with MBP, using an antibody raised against Erk-2.

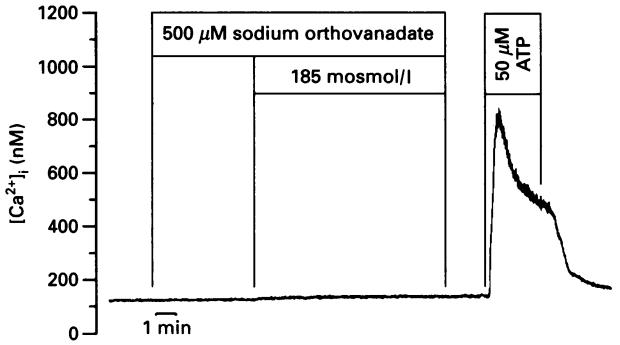

Figure 2 Effect of vanadate and hypo-osmolarity on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in H4IIE cells, measured with fura-2 on single cell level

H4IIE cells were loaded with 5 μM fura-2 acetylmethyl ester for 30 min. Following preincubation with 500 μM vanadate for 5 min, the medium osmolarity was reduced to 185 mosmol/l, maintaining the vanadate concentration constant. Vanadate and hypo-osmolarity did not influence $[Ca^{2+}]_i$ but 50 μM ATP caused a rapid and transient increase in $[Ca^{2+}]_i$. The trace is representative of five experiments.

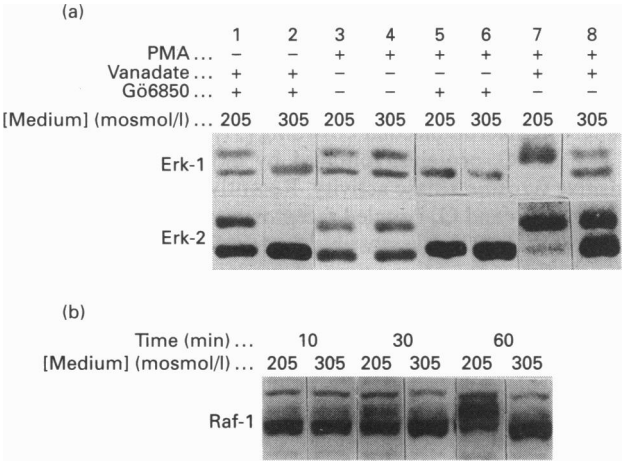

Figure 3 Activation of Erk-1 and Erk-2 in response to hypo-osmolarity is independent of PKC and does not depend on mobility-decreased Raf-1 in H4IIE cells

Representative results of at least three independent experiments are shown. Total protein was analysed by Western blots (**a**). Cells were treated as indicated: Gö6850 (1 μM), 20 min preincubation; vanadate (500 μM), present during 5 min preincubation and 10 min hypo- or normo-osmotic exposure; PMA (1 μM) present during 10 min hypo- or normo-osmotic incubation. Blots were probed with antisera raised against Erk-1 and Erk-2 respectively. (**b**) H4IIE cells were exposed to hypo- or normo-osmotic medium in the presence of 500 μM vanadate for the times indicated. The blot was probed with an antibody raised against Raf-1 kinase.

The swelling-induced phosphorylation of Erk-1 and Erk-2 is independent of PKC and does not involve mobility shifts of Raf-1

PKC-dependent and -independent routes of MAP-kinase activation have been described [19]. To examine whether the hypo-osmolarity-induced phosphorylation of Erk-1 and Erk-2 is dependent on the action of the PKC, Gö6850, a well characterized inhibitor of most PKC isoforms [20] was used. Figure 3(a) shows that 20 min pretreatment of the cells with 1 μM Gö6850 did not affect the phosphorylation of Erk-1 and Erk-2 after hypo-osmotic exposure (lanes 1 and 2) but abolished the Erk-phosphorylation caused by the PKC-activator phorbol 12-myristate 13-acetate (PMA, lanes 3–6). Thus, PKC is apparently not involved in upstream events leading to swelling-induced Erk activation.

PMA-induced activation of Erk-1 and Erk-2 seems unaffected by medium osmolarity (Figure 3a, lanes 3 and 4). In the presence of vanadate however, the inactive forms of Erk-1 and Erk-2 disappeared and only the activated Erk-1 and Erk-2 are detectable when the cells were exposed to hypo-osmotic conditions (lane 7). Under normo-osmotic control conditions vanadate had no effect: only the PMA-induced activation of MAP kinases was seen (lane 8). It is suggested, that the swelling-induced and the PMA-induced phosphorylation of MAP kinases result from activation of two different pathways, which appear to be additive.

Besides PKC, the Raf-1 kinase may be involved in the phosphorylation cascade, leading to activation of MAP kinases. The electrophoretic behaviour of Raf-1 kinase after hypo- or normo-osmotic exposure of the cells in the presence of vanadate was examined. Shifted Raf-1 kinase, which represents some phosphorylated forms of the protein, was not observed during the first 10 min of hypo-osmotic exposure. A slight increase in shifted Raf-1 kinase occurred after 30 min and became marked after 1 h (Figure 3b). It is possible that the MAP kinases act as Raf-1 kinase kinases. Whether the absence of shifted forms corresponds to the absence of hypo-osmolarity-induced activation of Raf-1 remains to be determined.

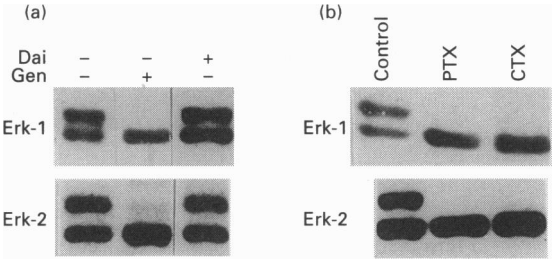

Figure 4 Hypo-osmolarity-induced activation of MAP kinases is sensitive to genistein, pertussis toxin and cholera toxin

Representative results of four independent experiments are shown. Cells were exposed to hypo- or normo-osmotic medium for 10 min in the presence of 500 μM vanadate. Total proteins were analysed by Western blot using antibodies raised against Erk-1 and Erk-2 respectively. (**a**) Genistein (Gen, 100 μM) and daidzein (Dai, 100 μM), 3 h pretreatment. (**b**) Pertussis toxin (PTX) (100 ng/ml), 20 h pre-treatment, cholera toxin (CTX, 5 μg/ml) 8 h pretreatment.

Inhibition of the swelling-induced activation of Erk-1 and Erk-2 by the tyrosine kinase inhibitor genistein and the G-protein modulators pertussis and cholera toxin

In order to examine the participation of tyrosine kinases in swelling-induced Erk activation, we used genistein, a known tyrosine kinase inhibitor [21]. Figure 4(a) shows that pretreatment of the cells with 100 μM genistein completely abolished the swelling-induced phosphorylation of Erk-1 and Erk-2, but in the presence of its inactive structure analogue, daidzein, the shifting of the Erk-proteins in response to swelling was not affected. Thus, genistein here specifically acts as a tyrosine kinase inhibitor, indicating that upstream of the MAP kinases a tyrosine kinase activity is involved in the swelling-stimulated signalling pathway.

To evaluate the involvement of G-proteins in the swelling-induced signalling, the cells were preincubated with pertussis or cholera toxin. Both toxins abolished the phosphorylation of Erk-1 and Erk-2 in response to cell swelling, suggesting that the

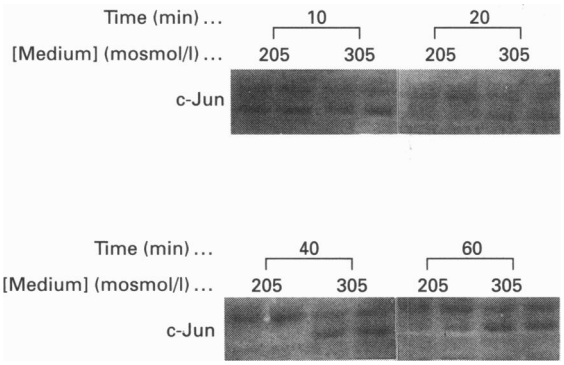

Figure 5 Hypo-osmolarity induces an increase in c-Jun phosphorylation

The results of two of at least four identically performed experiments are shown. Cells were exposed to hypo- or normo-osmolar medium in the presence of vanadate for the times indicated. Total protein was blotted and analysed using an antibody raised against c-Jun.

swelling-activated signal transduction pathway is sensitive to inhibition of both G_s- and G_i-proteins (Figure 3b).

Swelling-induced activation of Erk-1 and Erk-2 leads to a transient increase in c-Jun phosphorylation

We explored the changes in the phosphorylation state of c-Jun in response to cell swelling. Figure 5 shows a transient increase in retardation of the c-Jun polypeptide between 20 and 60 min under hypo-osmotic but not under normo-osmotic conditions in the presence of vanadate. The shifted protein corresponds to a phosphorylation state of c-Jun which is related to autoinduction of c-Jun transcription and to its transactivating function within the activating protein-1 complex [22]. This is in accordance with the about 5-fold increase of *c-jun* mRNA in response to cell swelling observed in H4IIE cells [10].

DISCUSSION

Cell-volume signalling

In this study data are presented which indicate activation of the MAP kinases Erk-1 and Erk-2 in response to hypo-osmotic cell swelling. This activation is sensitive to genistein as well as pertussis and cholera toxin, suggesting that cell swelling leads to a G-protein and tyrosine kinase-mediated activation of MAP kinase. Calcium and PKC are apparently not involved in the upstream events which lead to the swelling-induced Erk-activation.

The swelling-induced Erk activation is most likely a weak short-term signal. Its reproducible detection depends on the presence of vanadate. Vanadate prevents inactivation of Erk-1 and Erk-2 through its action as an inhibitor of the protein phosphatases 1B and stress activated CL100, which are responsible for dephosphorylation of Tyr-185 and both Thr-183 and Tyr-185 [23]. Recently it was suggested that vanadate may activate MAP kinases in CHO cells itself by inducing a calcium signal resulting from an inhibition of the Ca^{2+}/Mg^{2+}-ATPase [24]. Indeed some activators elicit the mobilization of intracellular calcium sources, which mediates Erk activation [25]. However, in H4IIE cells vanadate did not increase the phosphorylation of Erk-1, Erk-2 or c-Jun under normo-osmotic conditions. Moreover, no changes in intracellular free calcium in response to vanadate and hypo-osmolarity were detectable at the single-cell level. Thus, in H4IIE cells vanadate does not interfere with the swelling-induced activation of MAP kinases, which occurs without the requirement of calcium signalling.

The swelling- but not the PMA-induced activation of Erk-1 and Erk-2 is completely maintained in the presence of Gö6850, and thus indicates PKC-independence of the swelling-induced signalling (Figure 3a). Co-stimulation of the cells with PMA and hypo-osmolarity in the presence of vanadate caused the almost complete phosphorylation of Erk-1 and Erk-2, suggesting the additivity of two independent pathways. These two pathways possibly differ regarding the requirement of Raf-1 kinase for Erk activation. PKC_α is known to activate Raf-1 kinase by direct phosphorylation [26], the latter enzyme may then phosphorylate the MEK. Mobility-shifted Raf-1 is not involved in the upstream events, which are triggered by hypo-osmolarity to induce MAP kinase activity. However, a shift-inducing Raf-1 phosphorylation was observed, if cells were exposed to hypo-osmolarity for 1 h (Figure 3b), suggesting that Raf-1 possibly serves as substrate for MAP kinases. This corresponds to other reports, indicating that Raf-1 kinase is not the major upstream regulator of MAP kinases [27,28]. Also in the case of insulin signalling, evidence has been given that Erk-1 and Erk-2 act as a insulin-activated Raf-1 kinase kinases [29]. To decide the participation of Raf-1 in the signalling cascade induced by cell swelling requires the measurement of activity.

The swelling-induced MAP kinase activation is sensitive to genistein as well as to both pertussis and cholera toxin. It is possible that cell swelling-induced membrane stretch [30] or changes in macromolecular crowding [31] may trigger activation of G_i- and G_s-proteins, followed by induction of a genistein-sensitive tyrosine kinase activity. Recently Faure et al. reported, that both G_i- and G_s-coupled receptors can activate Erk proteins in COS-7 cells [32]. It is possible that swelling-induced signalling requires the cooperative action of subunits derived from several types of G-proteins on effector proteins, as discussed for other cases [33].

Recently evidence has been presented that hyper-osmotic exposure of mammalian cells induces the activation of MAP kinases, which differ from Erk-1 and Erk-2 with respect to structural features, evolutionary distance and the pattern of substrate specificity [34–36]. They are related to the yeast protein kinase high-osmolarity glycerol response 1 (HOG1), which is essential for growth on medium of high osmolarity [37]. Moreover they mediate the cellular response to heat shock and lipopolysaccharide but they are not stimulated by growth factors. In these studies no activation of Erk-1 and Erk-2 in response to hyper-osmotic shock could be demonstrated. This agrees with the lack of Erk activation we observed within at least 20 min of hyper-osmotic exposure in H4IIE cells, even when vanadate was present (results not shown). Furthermore, human Erk-2 was not able to complement the null mutant *hog*-1 of *Saccharomyces cerevisiae* [34]. Apparently cell swelling and cell shrinkage can activate different sets of proteins from the MAP kinase family. Nevertheless the activation of Erk-2 but not Erk-1 in the case of extremely hyper-osmotic exposure of renal Madin Darby canine kidney cells (400–600 mosmol/l) has recently been reported [38].

Functional significance

Erk-1 and Erk-2 are key elements in growth factor signalling [15]; their activation in response to hypo-osmotic cell swelling in H4IIE cells suggests that they play a central role in linking hepatocellular hydration to liver function. Thus, cell swelling induces transient phosphorylation of the c-Jun polypeptide following the activation of Erk-1 and Erk-2. Recently, evidence

was given that c-Jun is a substrate of Erk-1 and Erk-2 not only *in vitro* but also *in vivo* [39,40]. The phosphorylation of Ser-63 and Ser-73 within the N-terminal domain of c-Jun stimulates its transactivating potency and auto-induction of c-Jun expression [22]. Indeed an about 5-fold increase in *c-jun* mRNA levels under hypo-osmotic conditions in H4IIE cells has recently been reported [10]. Based on this, a pathway is postulated, which leads from swelling-induced Erk activation, followed by phosphorylation and auto-induction of c-Jun, to volume-sensitive regulation of gene expression. Another substrate of the MAP kinases is the S6 kinase ($p90^{rsk}$), which is involved in the control of protein and glycogen metabolism [41]. Indeed increased S6 phosphorylation, induced by swelling of primary hepatocytes has been reported [42]. Thus, the increase in protein biosynthesis following hepatocyte swelling [43], could result from phosphorylation of the ribosomal protein S6. Another substrate of the S6 kinase is protein phosphatase 1, whose Erk-mediated phosphorylation results in a selective increase in their glycogen-synthase-phosphatase activity [44]. Interestingly, cell swelling induces the liver glycogen-synthetase via induction of the synthase phosphatase activity [45]. Further evidence for the relevance of the swelling-activated signal transduction pathway presented here is given by recent findings with respect to the hypo-osmolarity-induced alkalinization of endocytotic vesicles in isolated hepatocytes, which is completely abolished in the presence of cholera and pertussis toxin as well as genistein, but not daidzein [18]. The same inhibiting pattern acts on the increase in taurocholate excretion in hypo-osmotically perfused rat liver (B. Noé, and D. Häussinger, unpublished work). Thus the swelling-induced signalling reported here, may represent, at least in part, the up to now missing link between cell hydration and cell function.

This work was supported by the Deutche Forschungsgemeinschaft, the Gottfried-Wilhelm-Leibniz Prize, the Schilling-Stiftung and the Fonds der Chemischen Industrie.

REFERENCES

1 Häussinger, D., Lang, F. and Gerok, W. (1994) Am. J. Physiol **267**, E343–E355
2 Häussinger D. and Lang, F. (1991) Biochim. Biophys. Acta **1071**, 331–350
3 Häussinger, D. and Lang, F. (1992) Trends Pharmacol. Sci. **13**, 371–373
4 Hallbrucker, C., Ritter, M., Lang, F., Gerok, W. and Häussinger, D. (1993) Eur. J. Biochem. **211**, 449–458
5 Saha, N., Schreiber, R., vom Dahl, S., Lang, F., Gerok, W. and Häussinger, D. (1993) Biochem. J. **296**, 701–707
6 Häussinger, D., Lang, F., Bauers, K.. and Gerok, W. (1990) Eur. J. Biochem. **188**, 689–695
7 Häussinger, D., Roth, E., Lang, F. and Gerok, W. (1993) Lancet **341**, 1330–1332
8 Newsome, W. P., Warskulat, U., Noe, B., Wettstein, M., Stoll, B., Gerok, W. and Häussinger, D. (1994) Biochem. J. **304**, 555–560
9 Lundgreen, D. V. (1992) J. Biol. Chem. **267**, 6841–6847
10 Finkenzeller, G., Newsome, W. P., Lang, F. and Häussinger, D. (1994) FEBS Lett. **340**, 163–166
11 Offensperger, W. B., Offensperger, S., Stoll, B., Gerok, W. and Häussinger, D. (1994) Hepatology **20**, 1–7
12 Schreiber, R., Stoll, B., Lang, F. and Häussinger, D. (1994) Biochem. J. **302**, 113–120
13 Busch, G. L., Schreiber, R., Dartsch, P. C., Völkl, H., vom Dahl, S., Häussinger, D. and Lang, F. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 9165–9169
14 Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., De Phino, R. A., Panayotatos, N., Cobb, M. H. and Yancopoulos, G. D. (1991) Cell **65**, 663–675
15 Davis, R. J. (1993) J. Biol. Chem. **268**, 14553–14556
16 Howe, L. R., Leevers, S. J., Gomez, N., Nakielny, S., Cohen, P. and Marshall, C. J. (1992) Cell **71**, 335–342
17 Samuels, M. L., Weber, M. J., Bishop, J. M. and McMahon, M. (1993) Mol. Cell. Biol. **13**, 6241–6252
18 Schreiber, R. and Häussinger, D. (1995) Biochem. J. **309**, 19–24
19 Chao, T. S., Foster, D. A., Rapp, U. R. and Rosner, M. R. (1994) J. Biol. Chem. **269**, 7337–7341
20 Martiny-Baron, G., Katzanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D. and Schächtele, C. (1993) J. Biol. Chem. **268**, 9194–9197
21 Hill, T. D., Dean, N., Mordan, L. J., Lau, A. F., Kanemitsu, M. Y. and Boynton, A. L. (1990) Science **248**, 1660–1663
22 Angel, P., Hattori, K., Smeal, T. and Karin, M. (1988) Cell **55**, 875–885
23 Alessi, D., Smythe, C. and Keyse, S. (1993) Oncogene **8**, 2015–2020
24 D'Onofrio, F. D., Le, M. Q. U., Chiasson, J.-L. and Srivastava, A. K. (1994) FEBS Lett. **340**, 269–275
25 Chao, T. S., Byron, K. L., Lee, K. M., Villeral, M. and Rosner, M. R. (1992) J. Biol. Chem. **267**, 19876–19883
26 Sözeri, O., Vollmer, K., Liyanage, M., Frith, D., Kour, G., Mark, G. E. and Stabel, S. (1992) Oncogene **7**, 2259–2262
27 Kondoh, S. K. and Okayama, H. (1993) FEBS Lett. **336**, 255–258
28 Anderson, N. G., Li, P., Marsden, L. A., Williams, N., Roberts, T. M. and Sturgill, T. W. (1991) Biochem. J. **277**, 573–576
29 Lee, R., Cobb, M. H. and Blackshear, P. J. (1992) J. Biol. Chem. **267**, 1088–1092
30 Sarcadi, P. and Parker, J. C. (1991) Biochim. Biophys. Acta **1071**, 407–427
31 Minton, A. P., Colclasure, G. C. and Parker, J. C. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10504–10506
32 Faure, M., Voyno Yasenetskaya, T. A. and Bourne, H. R. (1994) J. Biol. Chem. **269**, 7851–7854
33 Hepler, J. R. and Gilman, A. G. (1992) Trends Biochem. Sci. **17**, 383–387
34 Galcheva-Gargova, Z., Derijard, B., Wu, I.-H. and Davis, R. J. (1994) Science **265**, 806–808
35 Han, J., Lee, J. D., Bibbs, R. and Ulevitch, J. (1994) Science **265**, 808–811
36 Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A. R. (1994) Cell **78**, 1027–1037
37 Brewster, J. L., deValoir, N. D., Dwyer, E., Winter, E. and Gustin, C. (1993) Science **259**, 1760–1763
38 Terada, Y., Tomita, K., Homma, M. K., Nonoguchi, H., Yang, T., Yamada, T., Yuasa, Y., Krebs, E. G., Sasaki, S. and Marumo, F. (1994) J. Biol. Chem. **269**, 31296–31301
39 Bernstein, L. A., Ferris, D. K., Colburn, N. H. and Sobel, M. E. (1994) J. Biol. Chem. **269**, 9401–9404
40 Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E. and Woodgett, J. R. (1991) Nature (London) **353**, 670–674
41 Blenis, J. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 5889–5892
42 Luiken, J. J. F. P., Blommaart, E. F. C., Boon, L., van Woerkom, G. M. and Meijer, A. J. (1994) Biochem. Soc. Trans. **22**, 508–511
43 Stoll, B., Gerok, W., Lang, F. and Häussinger, D. (1992) Biochem. J. **287**, 217–222
44 Dent, P., Lavoinne, A., Nakielny, S., Candwell, F. B., Watt, P. and Cohen, P. (1990) Nature (London) **348**, 302–308
45 Meijer, A. J., Baquet, A., Gustafson, L., van Woerkom, G. M. and Hue, L. (1992) J. Biol. Chem. **267**, 5823–682

Received 13 January 1995/27 February 1995; accepted 2 March 1995