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Activation and nuclear translocation of ERK in response to ligand-dependent and -independent stimuli in liver and gill cells from rainbow trout

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Summary

The mitogen-activated protein kinase ERK is an important signalling molecule involved in the control of cell proliferation, differentiation and cell death, targeting molecules at the cell membrane, in the cytosol, and in the nucleus. This study investigated the activation pattern and subcellular distribution of ERK in liver and gill cells of rainbow trout upon hypo-osmotic shock, addition of epidermal growth factor (EGF) and copper treatment. It further set out to characterize the hypothetical role of nuclear-export signal (NES)-dependent relocation of ERK after nuclear entry and the potential involvement of the ERK activator MEK. Although, in primary hepatocytes, ERK was activated in all conditions in a stimulus-specific manner, it did not accumulate in the nucleus, irrespective of the absence or presence of the inhibitor of NES-dependent export leptomycin B (LB). Similarly, in trout hepatoma cells, where pERK levels increased upon osmotic and mitotic stimulation, but not after toxic insult, no significant nuclear translocation was observed. In a gill cell line, levels of pERK increased after osmotic and mitotic stimulation and showed a decrease during incubation with a toxicant. Again, none of these conditions triggered nuclear accumulation of pERK in the gill cells in the absence of LB, but in contrast to the observation in liver cells, both osmotic and mitotic stimulation caused nuclear accumulation in the presence of the inhibitor. The ERK activator MEK, which possesses a NES-sequence, was apparently not involved in nuclear export, as it did not seem to enter the nucleus. Altogether, ERK is activated in trout cells in a stimulus- and cell type-specific manner, and our data suggest that it acutely acts primarily on cytoplasmic or membrane-situated targets in liver cells, whereas it presumably triggers rapid transcriptional activities in gill cells.

Key words: trout hepatocyte, RTH-149, RTgill-W1, extracellular signal regulated kinase, nuclear translocation, hypo-osmolarity, copper, epidermal growth factor.

Introduction

Mitogen-activated protein (MAP) kinases are serine/threonine kinases activated in response to a variety of stimuli that mediate signal transduction from the cell surface to the nucleus as well as to cytoplasmic and membrane targets (de Nadal et al., 2002; Rosseland et al., 2005). The best studied MAP kinase pathway is that of the extracellular signal regulated protein kinases ERK1/2. ERKs are the terminal enzymes in a cascade of three protein kinases [MAPKKK (Raf), MAPKK (MEK) and MAPK (ERK)], which sequentially activate their downstream target by phosphorylation at specific amino acid residues. In addition to phosphorylating membrane-associated and cytosolic proteins, ERK is capable of translocating to the nucleus, where it regulates gene expression by activating transcription factors such as Elk-1, c-Fos, or c-Myc (Roux and Blenis, 2004). ERK has more than 70 recognized substrates, underpinning its important role in cellular signalling (Kolch, 2005). MEK1/2 are considered both direct activators as well as cytoplasmic anchoring proteins for ERK (Fukuda et al., 1997b; Grewal et al., 2006). In contrast to ERK itself (Pouysségur et al., 2002), MEK1/2 proteins typically possess a nuclear export signal (NES) sequence, which is a short leucine-rich region presumed to be essential for the fast nuclear export in some cells (Fukuda et al., 1997a). Based on these features, MEK1/2 may play a dual role in the control of ERK activity. On the one hand, by tethering ERK in the cytoplasm in non-stimulated cells, MEK prevents its nuclear translocation and thereby prohibits its action on nuclear target molecules. On the other hand, upon activation and nuclear translocation of ERK, MEK may itself enter the nucleus, re-associate with ERK and contribute to the nuclear export of ERK (Adachi et al., 2000). Although recent reports do not support the latter role of MEK (Burack and Shaw, 2005; Horgan and Stork, 2003), it has become increasingly clear that the subcellular localization of ERK is under complex control, as it is critical for its stimulus-specific action on its diverse targets.

In a recent study on the role of ERK in the response of trout

hepatocytes to aniso-osmotic stress we observed that, although ERK was rapidly activated upon hypo-osmotic exposure, it did not enter the nucleus of the cells. Even after inhibition of NES-dependent export, to prevent the above-described hypothetical mechanism, ERK did not accumulate in the nucleus. This suggested that, after hypo-osmotic activation of the kinase, its main site of action was in the cytoplasm or at the cell membrane (Ebner et al., 2006). In line with this notion we observed that inhibition of ERK activation significantly delayed cell volume recovery, a process that takes place within a time-scale of approximately 40 min and will thus most likely not rely on transcriptional activities triggered by ERK. Similar findings were obtained upon inhibition of p38-MAPK in the trout cells (Ebner et al., 2006) as well as upon inhibition of MAPKs in rat liver (vom Dahl et al., 2001) and in amphibian renal A6 cells (Chiri et al., 2004). This indicates that MAPKs play an important role in the acute response to osmotic stress. At the same time, however, numerous studies have shown that MAPKs are important regulators of adaptive responses to prolonged osmotic stress, where the induction of gene transcription is of pivotal importance (de Nadal et al., 2002).

In the present study we thus addressed the question of whether the apparent absence of nuclear translocation of ERK in trout hepatocytes was indeed related to the specific stimulus imposed on the cells (i.e. hypo-osmotic stress), or if it was rather due to the cell type investigated (i.e. liver cells) or the condition of the cells (i.e. primary hepatocytes). In order to study the stimulus-specificity of the ERK response we investigated if the induction of metal stress caused by exposure to copper, which dose-dependently activates ERK in trout hepatocytes (Nawaz et al., 2006), would elicit nuclear translocation, or if a classical mitogenic stimulus such as application of epidermal growth factor (EGF) could induce the nuclear entry of ERK. In addition, to elucidate the cell-type specificity of ERK activation and subcellular distribution, the responses to the same stimuli were also studied in a trout hepatoma cell line as well as in a gill cell line. Finally, we examined if under any of these conditions NES-dependent export would play a role in the subcellular localization of the MAPK. Since a previous study reported that, in contrast to mammals, a MEK isoform of carp lacks the NES-sequence (Hashimoto et al., 2002), we cloned and sequenced MEK from trout hepatocytes in order to see if this was also true for this fish species and could thus be related to the subcellular distribution patterns observed.

Materials and methods

Materials

Collagenase (Type VIII), bovine serum albumin (BSA), fetal bovine serum (FBS), poly-L-lysine (PLL), epidermal growth factor (EGF), horseradish peroxidase (HRP)-conjugated monoclonal anti rabbit secondary antibody for western blot analysis, and propidium iodide were all purchased from Sigma (Deisenhofen, Germany). Leibovitz L-15, MEM medium, non-essential amino acids, antibiotics and sodium pyruvate were bought from Invitrogen (Vienna, Austria). Leptomycin B (LB) was bought from LC Laboratories (Woburn, MA, USA). MAP kinase antibodies were obtained from Cell Signaling Technology (New England Biolabs, Beverly, MA, USA), FITC-labelled secondary antibody for immunocytochemistry was from DAKO (Glostrup, Denmark), and Vectashield mounting medium was from Vector Laboratories (Burlingame, CA, USA). All other chemicals were of analytical grade and purchased from local suppliers.

Culture of primary hepatocytes and of hepatoma (RTH-149) and gill cell lines (RTgill-W1)

Hepatocytes for primary cultures were obtained from rainbow trout *Oncorhynchus mykiss*, purchased from a local hatchery (Thaur, Tyrol). Animals were fed daily *ad libitum* with trout pellets and kept at 15°C in well-aerated 200-l aquaria. Fish were sacrificed by a sharp blow on the head and transsection of the spinal chord, and the liver was perfused to remove the blood. Collagenase digestion and differential centrifugation were applied to isolate hepatocytes as described previously (Krumschnabel et al., 1996). Following isolation, cells were suspended in standard medium containing 1% BSA (see experimental media) and left to recover at 19°C for 1 h in a water bath shaking at 120 r.p.m. Cells were then counted in a Bürker–Türk hemocytometer and viability was determined by the Trypan Blue exclusion method. For hepatocytes used in this study, cell viability was always above 95%. For western blot analysis 10^7 cells were seeded onto untreated 3.5 cm Petri dishes, whereas for immunocytochemistry 2×10^6 hepatocytes were seeded onto PLL-coated glass coverslips in similar Petri dishes. Hepatocytes were then cultured overnight in an incubator at 19°C and 0.5% CO_2 with L-15 medium containing 10 mmol l^{-1} Hepes, 5 mmol l^{-1} $NaHCO_3$, 50 μg ml^{-1} gentamicin, 100 μg ml^{-1} kanamycin, titrated to a final pH of 7.6. RTH-149 and RTgill-W1 cell lines were bought from ATCC (Manassas, VA, USA) and established as indicated by the supplier. After a first propagation of RTH-149 cultures in modified MEM medium containing 1% penicillin–streptomycin, non-essential amino acids and sodium pyruvate, cells were slowly adapted to L-15 medium with 10% FBS and 1% penicillin–streptomycin and further cultured at 19°C in an air atmosphere. The same medium and culture conditions were used for RTgill-W1 cells. For immunocytochemistry these cell lines were then grown on PLL treated glass coverslips in small Petri dishes. For western blot analysis they were grown on 100×20 mm tissue culture dishes. Harvesting of the cell cultures was conducted at approximately 75% of confluence. Examination of cell viability in the permanent cell lines by Trypan Blue after a medium exchange, which always preceded experimental exposures, indicated that no dead cells were present in these cell cultures.

Experimental media

The standard incubation medium consisted of (in mmol l^{-1}) 10 Hepes, 136.9 NaCl, 5.4 KCl, 1 $MgSO_4$, 0.33 NaH_2PO_4, 0.44 KH_2PO_4, 1.5 $NaHCO_3$, 1.5 $CaCl_2$, 5 glucose and 10% FBS with

a pH adjusted to 7.6 and an osmolarity of 284 mOsmol l^{-1}. Hypo-osmotic conditions were created by mixing equal volumes of standard saline with the same medium lacking NaCl, yielding 0.58×iso-osmolarity (165 mOsmol l^{-1}). Medium osmolarities were assessed by freezing point depression using a Knaus Semi-Micro Osmometer (Berlin, Germany). For assessment of the effects of LB, an inhibitor of nuclear export signal-dependent transport, cells were pre-incubated with 0.4 ng ml^{-1} of the inhibitor for 60 min, a condition previously reported to be effective in other cell types (Adachi et al., 2000). Following this pre-incubation period half of the iso-osmotic saline was replaced by an equal volume of medium lacking NaCl (hypo-osmotic conditions), or iso-osmotic saline containing EGF or Cu^{2+} to obtain a final concentration of 30 nmol l^{-1} or 10 μmol l^{-1}, respectively. A concentration of 30 nmol l^{-1} EGF was chosen, as preliminary experiments indicated a clear and reproducible response of the cells to this concentration for all cell types. The choice of 10 μmol l^{-1} Cu^{2+} was based on the previous observation that it caused a significant activation of ERK in trout hepatocytes (Nawaz et al., 2006), but did not induce significant cell death in any of the cell types used over the time of incubation studied (data not shown). In each case, the switch from culture medium to experimental saline occurred immediately before experimental exposure. In order to exclude the possibility that this acute change of medium affected either the activity or sub-localization of ERK, non-stimulated control cultures were run in parallel for each treatment after medium exchange. These experiments indicated that neither the levels of phosphorylated ERK detected by western blot analysis nor the cellular compartmentalization of the kinase were affected by the medium exchange.

Protein extraction and western blot analysis

Following exposure of the cells to different conditions for the required period, primary hepatocytes were collected by vigorous pipetting, but cell lines were harvested by scraping the cells off the substrate. Cells were then rapidly spun down by brief centrifugation, the medium removed by aspiration, and pellets broken up in liquid nitrogen and lysed in a buffer containing 25% glycerol, 420 mmol l^{-1} NaCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} EDTA and 20 mmol l^{-1} Hepes, with 0.5 mmol l^{-1} dithiothreitol (DTT) and 0.5 mmol l^{-1} phenylmethyl sulphonyl fluoride (PMSF) added freshly before use. Cell extracts were subsequently briefly centrifuged and total protein collected from the supernatants was stored at –80°C. Protein content of the samples was determined by use of the colorimetric Bio-Rad DC Protein Assay and aliquots containing 30 μg of protein were electrophoretically separated on a NuPage™ system 10% Bis-Tris-Gel (Invitrogen) in NuPage® MOPS SDS Running Buffer (Invitrogen) at 200 V for 60 min. Proteins were then transferred onto an Immun-Blot™ PVDF Membrane (BioRad) at 25V 60 min^{-1} in an XCell2™ Blot Module (Invitrogen) using NuPage® Transfer Buffer (Invitrogen). The membrane was then washed for 5 min with Tris buffered saline (TBS), followed by blocking for 2 h at room temperature with 1× TBS-T (1× TBS with 0.1% Tween-20) containing 5% non-fat dry milk powder. Total ERK, phosphorylated ERK (pERK) and phosphorylated MEK (pMEK) were detected by incubating the membrane overnight at 4°C with appropriate primary antibodies diluted 1:1000 in 1× TBS-T and 5% BSA. After several washes with TBS-T, HRP-linked, anti-rabbit secondary antibody was added at 1:5000 in blocking buffer and the membrane incubated for another 60 min at room temperature. Finally bands were visualized by incubating the membranes in ECL Western Blotting Detection Reagents (Amersham Bio Sciences Europe, Vienna, Austria) for 1 min and subsequent exposure to a high performance chemiluminescence film (Hyperfilm ECL, Amersham Bio Sciences). Following scanning of the film, protein abundance was then analysed by densitometry using Quantity One (BioRad) software.

In order to assess if any of the treatments would affect abundance of total ERK (i.e. non-phosphorylated and phosphorylated ERK), we also determined total ERK levels for each condition. As total ERK abundance did not change, this then also served to monitor equal loading of proteins on the gels. In addition, equal protein loading was checked by staining gels with Coomassie Blue right after protein transfer and visual inspection of high molecular protein bands retained on the gels.

Staining and imaging of cells for confocal laser scanning microscopy

For immunocytochemical staining of pERK and pMEK in cells after experimental exposure, cultures were rinsed once with phosphate-buffered saline (PBS, pH 7.6) and immediately fixed in a 4% solution of paraformaldehyde in PBS for 60 min at room temperature. Following permeabilization with PBS containing 0.1% Triton X-100 (PBS-T) for 30 min, non-specific protein staining was prevented by blocking for 1 h with PBS-T containing 1% BSA and 10% FBS. Subsequently cells were incubated overnight in a humidified dark chamber at +4°C with phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (pERK1/2) at a dilution of 1:200 or with pMEK (Ser217/221) antibody diluted 1:1000. Following three further wash steps in PBS-T, a secondary FITC-labelled anti-rabbit antibody (1:150) was applied, and cells were incubated in a dark chamber for 1 h at room temperature. For staining of the nucleus, an incubation step of 2 min with 5 μmol l^{-1} propidium iodide was introduced after incubation with the secondary antibody. Following three additional washing steps with PBS-T the staining was stabilized by embedding the cells in Vectashield mounting medium and the coverslips were fixed on object slides with commercially available nail polish. Visualization was performed on a confocal laser scanning fluorescence microscope (LSM 510, Zeiss, Axiovert100M) at the appropriate excitation and emission wavelengths. Images were captured by use of Zeiss physiology software package version 3.2 and processed with LSM 5 Image Browser (Zeiss, Vienna, Austria).

In order to obtain a quantitative estimate for the extent of nuclear translocation of pERK, captured images of cells were

then analyzed by calculating the ratio of staining intensity measured in the nucleus to that of a cytoplasmic region of the cell. Using this procedure, problems arising due to variability in staining efficiency between individual cell cultures could be eliminated.

Sequencing of MEK1/2

Total RNA was isolated from freshly isolated trout hepatocytes using TRIZol reagent (Invitrogen) and transcribed into cDNA with PowerSript reverse transcriptase (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). To obtain the central DNA region of trout MEK1/2, primers designed for the conserved region of MEK1/2 sequence of related species were applied and 5′- and 3′ ends were extended with nested rapid amplification of cDNA ends (RACE) PCR using gene-specific primers and adapter primers from a SMART^{TM} RACE cDNA Amplification Kit (Takara Bio Europe/Clontech). The PCR products were cloned into a pCR4-TOPO-vector, propagated in TOPO10 *E. coli* cells (TOPO TA Cloning Kit for Sequencing, Invitrogen), and sub-cloned. The plasmid DNA was purified with a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and sequenced using Big Dye Terminator v1.1 cycle sequencing reagents and M13 primers on an ABI Prism 373 (Applied Biosystems, Warrington, UK) automated DNA sequencer. All obtained sequences were examined by BLAST analysis for gene identification and for sequence similarities. Evaluation of the sequences obtained was performed using the GeneRunner program v3.0 (Hastings software), and multiple sequence alignments were performed using ClustalW.

Data presentation

Images and western blots presented are examples obtained in different experiments on at least three independent cell cultures. In the case of primary hepatocytes, cultures obtained from three different cell preparations were used. Statistical differences were evaluated applying unpaired Student's *t*-test (nuclear/cytoplasmic ratio with and without leptomycin B) or analysis of variance (western blot time series and time series of changes in nuclear/cytoplasmic ratio) followed by Student–Newman–Keuls test, with a $P<0.05$ being considered significant.

Results

Sequence analysis of trout cDNA encoding MEK

Sequencing of the coding region of the MEK gene expressed in rainbow trout hepatocytes yielded a product of 1197 base pairs, which translate into a protein of 399 amino acids (Fig. 1, GenBank accession number AJ966781). The amino-terminal end includes a leucine-rich NES sequence from amino acid 36–47, thought to be involved in the active nuclear export of ERK1/2 (Adachi et al., 2000; Kondoh et al., 2005). Furthermore, a putative MAPK docking site of 27 amino acids and a catalytic domain of 295 amino acids were detected. Blast analysis indicated highest sequence homology of trout MEK with MEK1 of carp, the nucleotide sequence identity amounting to 57% and the amino acid sequence identity to 90%, whereas identities of 44% and 30%, respectively, were obtained in comparison with the carp cMKK4 isoform that does not contain a NES sequence (Fig. 1). Comparisons with MEK isoforms from other vertebrates and an invertebrate species yielded amino acid sequence identities of 85% (*H. sapiens* MAP2K2), 84% (*R. norvegicus* MAP2K2), 79% (*M. musculus* MAPKK1), and 58% (*D. melanogaster* MEK1/2).

Compartmentalization of ERK

Primary cultures of trout hepatocytes

In trout hepatocytes, ERK was activated within 5 min of hypo-osmotic exposure and the levels of phosphorylated ERK (pERK) remained elevated over the entire 60 min period (Fig. 2A). In accordance with previous studies (Ebner et al., 2006; Nawaz et al., 2006) total ERK levels were not affected over this time of experimental exposure, and the same was true for the other treatments as well in both cell lines used (not shown). According to microscopic image analysis, activated ERK was locally restricted to the cytoplasm of trout hepatocytes, where it partly formed highly fluorescent spots, but it was clearly excluded from the nucleus (Fig. 3A). This distribution pattern was unaltered in hepatocytes that had been pre-incubated with LB, an agent known to block NES-dependent nuclear export, before hyposmotic exposure (Fig. 3A). A slightly different response pattern was obtained following ligand-dependent stimulation of hepatocytes with 30 nmol l^{-1} of EGF. In this case, western blot analysis detected a reproducible biphasic increase of pERK levels after addition of EGF (Fig. 2A). Immunocytochemical detection again indicated cytosolic localization of pERK with only little fluorescence in the nucleus (Fig. 3A). Pre-incubation of hepatocytes with LB tended to prolong ERK activation as well as to stabilize it, but again no nuclear accumulation of the MAPK could be detected. In a third experimental series, the impact of 10 μmol l^{-1} Cu^{2+} on ERK activity and localization was tested in trout hepatocytes. As shown in Fig. 2A, this caused a comparatively moderate, but persistent increase in pERK starting after 5 min of Cu^{2+}-exposure. Confocal laser scanning images revealed the absence of significant nuclear translocation and this was again unchanged by the presence of LB (Fig. 3A).

In order to elucidate whether these results were specific to the cell type investigated or could be attributed to the specific condition of the primary cell culture, we next examined ERK activity and translocation in a gill cell line and a hepatoma cell line from rainbow trout.

RTgill-W1 cell line

Following hypo-osmotic exposure of gill cells, ERK activity showed a slight, although not significant, increase within the first 2–5 min and had returned to a comparatively stable baseline level after 10 min (Fig. 2B). Addition of EGF elicited an elevation of pERK, which was transient in three out of four experiments and biphasic in one experiment. In contrast to these treatments, Cu^{2+} exposure of these cells consistently

caused no increase, but rather a transient decrease of pERK below the basal level seen in controls.

Immunocytochemical examination of the cells confirmed that both hypo-osmolarity and stimulation with EGF caused an increase of pERK levels, but in both cases pERK remained predominantly cytoplasmic (Fig. 3B). However, when these stimuli were imposed on the cells following pre-incubation with LB, they did cause nuclear accumulation of pERK, culminating in a maximal nuclear fluorescence signal after 30 min of hypo-osmotic exposure and at 5–15 min after EGF addition. As shown in Fig. 4A for EGF-treated cells, this resulted in an increase of the ratio of nuclear/cytoplasmic pERK staining from 0.7 to 1.8. Noteworthy, we observed that the initial ratio of 0.7 was already slightly, but significantly, elevated compared to that of 0.5 seen in cells in the absence of LB. Subsequently, despite the continuous presence of LB, pERK levels and the nuclear/cytoplasmic ratio diminished again, which is in line with the decrease of pERK seen in cell lysates. Differently from these treatments, but in agreement with the corresponding western blot data, Cu^{2+} exposure caused neither activation of ERK nor its nuclear translocation. Nevertheless, in LB-treated gill cells a slight, but significant increase of the nuclear/cytoplasmic signal ratio was elicited by Cu after 5 min, but unlike hypo-osmotic and EGF treatment this ratio did not exceed the value of 1. This indicates nuclear entry of pERK but no nuclear accumulation in this condition (Fig. 3B, Fig. 4B).

```
tMEK          ---MAPKRRPVP--LNITP-IGEGQSI-----------------------------STT 24
cMEK1         ---MAPKRRPVP--LIIAP-TGEGQ-------------------------------STN 22
hMAP2K2       ---MLARRKPVLPALTINPTIAEGP-------------------------------SPT 25
rMAP2K2       ---MLARRKPVLPALTINPTIAEGP-------------------------------SPT 25
cMKK4         MATSSPSSTPAASASSAQHHQTQSQHISTMSSMQDISSCWRFQSDSGKRKALKLNFANPP 60
                    .   *.          :.                                  ..

tMEK          IDAASEANLEALQKKLGELDLDEQQRKRLEAFLTQKAQVG---ELKDDDFHPICELGAGN 81
cMEK1         IDAASEANLEALQRKLGELDLDEQQRKRLEAFLTQKAQVG---ELKDEDFDPICELGAGN 79
hMAP2K2       SEGASEANLVDLQKKLEELELDEQQKKRLEAFLTQKAKVG---ELKDDDFERISELGAGN 82
rMAP2K2       SEGASEAHLVDLQKKLEELDLDEQQRKRLEAFLTQKAKVG---ELKDDDFERISELGAGN 82
cMKK4         IKPTSRITLNTAGLPFQNPHIERLRTHSIESSGKLKISPEQHWDFTAEDLKDLGEIGRGA 120
               . :*.  *      : : .::. : : :*:  . * .     ::. :*:. : *:* *

tMEK          GGVVNKVRHKPSRLVMARKLIHLEFKPAIRNQIIRELQVLHECN-SPYIVGFYGAFYSDG 140
cMEK1         GGVVHKVRHKPSRLVMARKLIHLEIKPAIRNQIIRELQVLHECN-SPYIVGFYGAFYSDG 138
hMAP2K2       GGVVTKVQHRPSGLIMARKLIHLEIKPAIRNQIIRELQVLHECN-SPYIVGFYGAFYSDG 141
rMAP2K2       GGVVTKARHRPSGLIMARKLIHLEIKPAVRNQIIRELQVLHECN-SPYIVGFYGAFYSDG 141
cMKK4         YGSVNKMVHKPSGQIMAVKRIRSTVDEREQKQLLMDLDVVMRSSDCPYIVQFYGALFREG 180
               * * *  *:**  :** * *:  ..   ::*:: :*:*: ... .**** ****:: :*

tMEK          EISICMEHMDGGSLDQVLKEARR-----IPEEILGKVSIAVLRGLAYLREKHQIMHRDVK 195
cMEK1         EISICMEHMDGGSLDQVLKEARR-----IPEEILGKVSIAVLRGLVYLREKHQIMHRDVK 193
hMAP2K2       EISICMEHMDGGSLDQVLKEAKR-----IPEEILGKVSIAVLRGLAYLREKHQIMHRDVK 196
rMAP2K2       EISICMEHMDGGSLDQVLKEAKR-----IPEDILGKVSIAVLRGLAYLREKHQIMHRDVK 196
cMKK4         DCWICMELMS-TSFDKFYKYVYSSLDEVIPEEILGKITLVTVKALNHLKENLKIIHRDIK 239
              :  **** *.  *:*:. * .       ***:****:::.::.* :*:*: :*:***:*

tMEK          PSNILVNSRGEIKLCDFGVSGQLIDSMANS-FVGTRSYMSPERLQG----THYSVQSDVW 250
cMEK1         PSNILVNSRGEIKLCDFGVSGQLIDSMANS-FVGTRSYMSPERLQG----THYSVQSDVW 248
hMAP2K2       PSNILVNSRGEIKLCDFGVSGQLIDSMANS-FVGTRSYMAPERLQG----THYSVQSDIW 251
rMAP2K2       PSNILVNSRGEIKLCDFGVSGQLIDSMANS-FVGTRSYMSPERLQG----THYSVQSDIW 251
cMKK4         PSNILLDRKGNIKLCDFGISGQLVDSIAKTRDAGCRPYMAPERIDPSASRQGYDVRSDVW 299
              *****:: :*:*******:****:**:*::  .* *.**:***::       *.*:**:*

tMEK          SMGLSLVELAIGRYPIPPPDAKELEAIFGRPVQDGAEGEPQAPSNRLPRPPGGRPVSGHG 310
cMEK1         SMGLSLVELAIGRFPIPPPDAKELEAIFGRAVLDKGGAEGHSMSPRQ-RPPG-RPVSGHG 306
hMAP2K2       SMGLSLVELAVGRYPIPPPDAKELEAIFGRPVVDGEEGEPHSISPRP-RPPG-RPVSGHG 309
rMAP2K2       SMGLSLVELAIGRYPIPPPDAKELEASFGRPVVDGADGEPHSVSPRP-RPPG-RPISGHG 309
cMKK4         SLGITLYELATGRFPYP------------------------------------------ 316
              *:*::* *** **:* *

tMEK          MDSRPAMAIFELLDYIVNEPPPRLPLG---VFTNDFQEFVTKCLIKNPAERADLKMLMNH 367
cMEK1         MDSRPAMAIFELLDYIVNEPPPKLPHG---VFTTDFEEFVMKCLMKNPADRADLKMLMGH 363
hMAP2K2       MDSRPAMAIFELLDYIVNEPPPKLPNG---VFTPDFQEFVNKCLIKNPAERADLKMLTNH 366
rMAP2K2       MDSRPAMAIFELLDYIVNEPPPKLPSG---VFSSDFQEFVNKCLIKNPAERADLKLLTNH 366
cMKK4         ----KWNSVFDQLTQVVKGDPPQLSSSEERQFSPKFINFVNLCLTKDESKRPKYKELLKH 372
                     ::*: *  :*:  **:*. .    *: .* :**  ** *: :.*.. * *  *

tMEK          TFIKR--AEEVDFAGWMCKTMGLNQPSTPTRITE- 399
cMEK1         TFIKRAEVEEVDFAGWMCKTMGLHQPSTPTHSAE- 397
hMAP2K2       TFIKRSEVEEVDFAGWLCKTLRLNQPGTPTRTAV- 400
rMAP2K2       AFIKRSEGEDVDFAGWLCRTLRLKQPSTPTRTAV- 400
cMKK4         PFIQMYEERTVDVASYVCKILDEMPASPSSPMYVD 407
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Fig. 1. Amino acid alignment of trout tMEK, deduced from the determined complete coding region of the nucleotide sequence, and carp cMEK1 isoform (GenBank accession no. AB063390), human hMAP2K2 (BC018645), rat rMAP2K2 (NM_205388), and carp cMKK4 (AB063389). Specific protein interaction sites indicated are: putative MAPK docking site (amino acids 1–27; underlined), NES-sequence (AA 36–47, boxed), and catalytic domain (AA 70–364, grey background); amino acid numbering refers to trout MEK.

Since our sequence analysis indicated that trout MEK has a NES-sequence and might thus be involved in the nuclear export of ERK, we tested if, under conditions where nuclear accumulation of pERK could be elicited, activated MEK would also enter the nucleus. The active rather than total form of MEK was determined, so as to visualize only the fraction of the kinase stimulated by the specific stimulus applied. However, despite a slight activation of the protein (Fig. 5A), no significant nuclear accumulation of pMEK could be observed following stimulation of gill cells with EGF, neither with nor without LB (Fig. 5B). Expressed as the nuclear/cytoplasmic signal ratio, a significant decrease could be detected as early as 2 min after stimulation. This mirrors the fact that although MEK is activated it remains largely localized to the cytoplasm. Similarly, we found no entry of pMEK into hepatocyte nuclei (not shown), suggesting that the absence of nuclear accumulation of ERK in these cells is not due to a rapid export by MEK.

RTH-149 cell line

In the trout hepatoma cell line hypo-osmolarity caused a transient increase of pERK levels within 5 min, followed by a return to near baseline values after 20 min (Fig. 2C). This

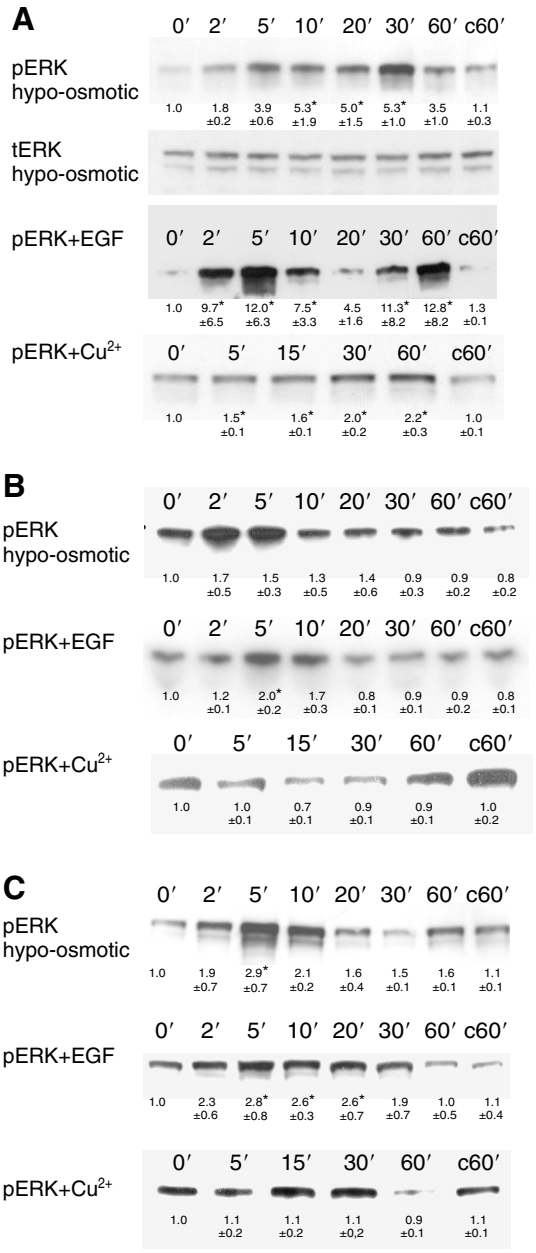

Fig. 2. Dynamics of ERK phosphorylation in primary hepatocytes (A), RTgill-W1 cells (B) and RTH-149 hepatoma cells (C) from rainbow trout exposed to hypo-osmolarity, 30 nmol l^{-1} EGF or 10 μmol l^{-1} $CuCl_2$, for up to 60 min as indicated. Numbers below the blots are changes in pERK abundance, expressed relative to controls at time zero, denoted as means ± s.e.m. of at least 3 independent experiments. *Statistically significant difference from the control value, as assessed on non-normalized data (P<0.05). c60′, controls incubated in standard conditions for 60 min. Activated ERK was determined by western blot analysis using an antibody against the dually phosphorylated form of ERK. The main band detected with this antibody migrated at approximately 44 kDa. In A, the second panel shows an example of a western blot analysis of total ERK abundance using an antibody against the phosphorylated and non-phosphorylated form of ERK (tERK). Similar analyses were made for all cell types under all conditions and indicated that ERK abundance was not altered by the treatments over the time course studied.

activation of ERK was also seen in immunocytochemical images, but in contrast to the gill cell line, even after pre-incubation with LB, no nuclear translocation of pERK was detected (Fig. 3C). Stimulation of hepatoma cells with EGF caused an increase of pERK levels within 2–5 min, and a return to basal levels after 60 min. Confocal images confirmed this transient activation pattern and indicated that in this case there was also no nuclear accumulation of ERK detectable, irrespective of the absence or presence of LB. Finally, we observed that upon Cu^{2+} exposure trout hepatoma cells showed no significant activation of ERK and no nuclear accumulation was observed either with or without LB.

Discussion

Activation and lack of translocation of ERK in hepatocytes

The present study was prompted by a recent observation that hypo-osmotic shock induced the activation, but not nuclear accumulation, of ERK in trout hepatocytes, and that inhibition of ERK activation interfered with volume regulation of these cells (Ebner et al., 2006). Together, these data suggested that the target molecules of the MAP kinase under these conditions were located at the cell membrane or in the cytoplasm, and that the effects of ERK on transcriptional activities described for other cell types exposed to aniso-osmotic conditions were not involved in the short-term regulatory function of the kinase. Our data not only confirmed these findings for hypo-osmotic conditions, but also showed a lack of nuclear pERK accumulation in trout hepatocytes upon stimulation of the MAP kinase with EGF or Cu^{2+}. As nuclear accumulation was still absent after inhibition of NES-dependent nuclear export with LB, this suggests that NES-dependent export is not important for relocation of ERK to the cytoplasm. In support of the latter it was suggested that ERK relocation is mainly due to passive diffusion (Burack and Shaw, 2005). In contrast, in the presence of LB, nuclear accumulation of pERK could be artificially enhanced in conditions where it was otherwise absent in the cells studied, i.e. upon EGF-stimulation (Whitehurst et al., 2004). The same is true for the gill cells examined here, where LB induced nuclear accumulation not only upon hypo-osmotic shock but also after addition of EGF. Thus, we believe that in the trout hepatocytes the stimuli applied did indeed fail to cause nuclear translocation of ERK within the time frame examined.

NES-dependent export and trout MEK

Unlike the absence of nuclear accumulation in hepatocytes, our data clearly show that in trout gill cells pERK entered the nucleus upon activation and was subsequently relocated to the cytosol *via* a NES-dependent transport mechanism. Analysis of the upstream activator MEK expressed in trout cells showed that this MAPKK possesses a NES-sequence and could thus in principle be involved in this process. However, our experiments using a pMEK-specific antibody indicated that even in LB-treated cells exposed to EGF there was no significant nuclear accumulation of pMEK. Thus, although the hypothesis of a MEK-mediated export of ERK has been

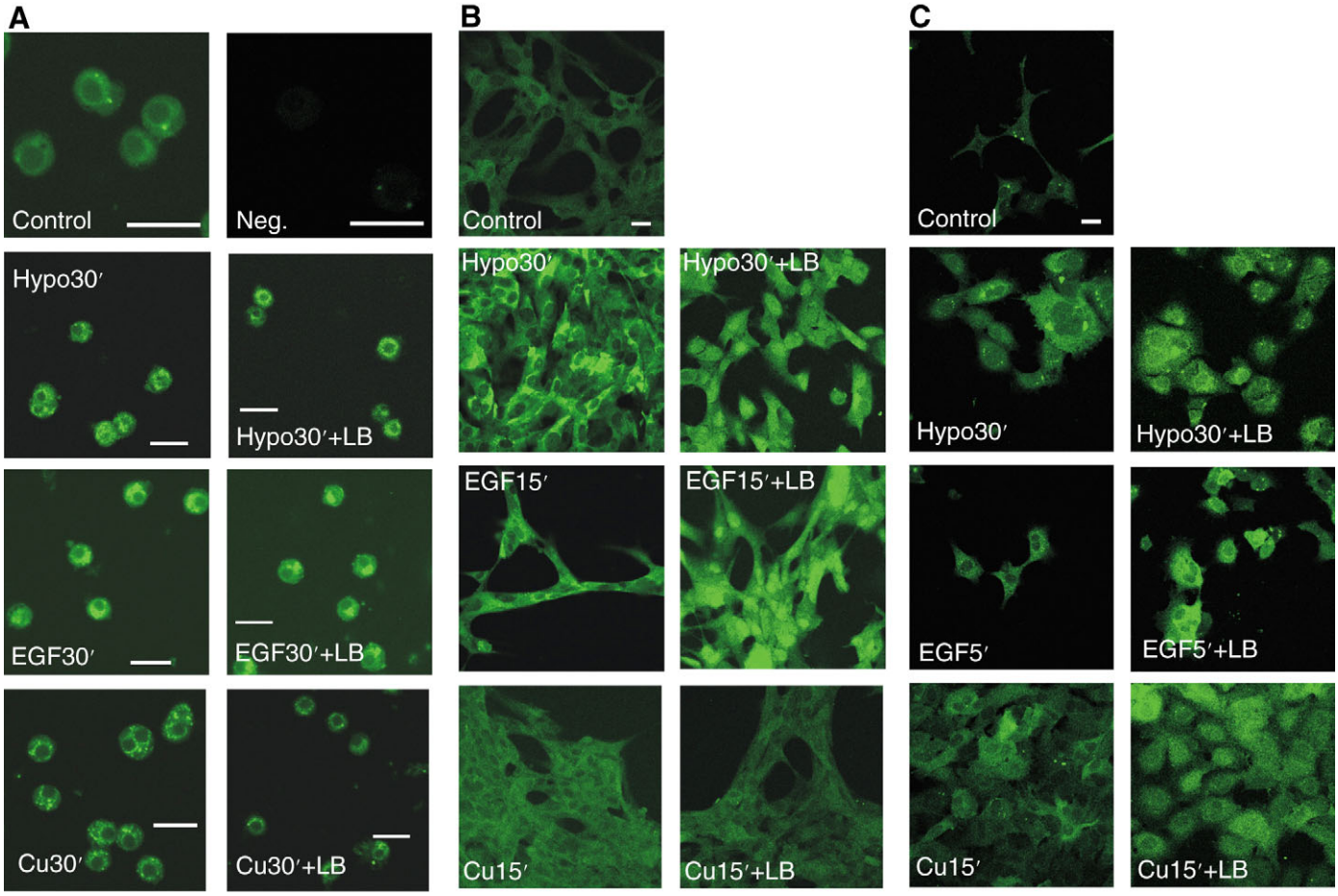

Fig. 3. Cellular distribution of phosphorylated ERK in primary hepatocytes (A), RTgill-W1 cells (B) and RTH-149 hepatoma cells (C) from rainbow trout stimulated by exposure to hypo-osmolarity (Hypo), EGF or Cu, in the absence or presence of leptomycin B (LB), as indicated. Cells were stained with an antibody against the dually phosphorylated form of ERK and a FITC-labelled secondary antibody and were then examined by confocal laser scanning microscopy. The right panel in the top row in A shows a negative control (Neg.) stained in the absence of the primary antibody. Scale bars, 20 μm (valid for all images in B and C).

recently reconfirmed in a complex mathematical modelling simulation (Fujioka et al., 2006), our data seem to support other studies indicating that the main role of NES-containing MEK is that of a cytoplasmic anchor for ERK, preventing its nuclear entry in the absence of an adequate stimulus (Fukuda et al., 1997b; Grewal et al., 2006).

It should be noted that besides MEK there are several other proteins involved in the control of the subcellular localization of ERK. It has, for example, been shown that ERK signalling to the nucleus may be anchorage-dependent, with integrin-mediated adhesion being required for entry of activated ERK into the nucleus (Aplin et al., 2001). The inhibitor of Ras/MAP kinase FGF signalling Sef, on the other hand, was reported to prevent ERK nuclear translocation by binding to activated MEK and inhibiting dissociation of the cytoplasmic MEK/ERK complex (Torii et al., 2004). Finally, it was shown that the phosphoprotein enriched in astrocytes 15 kDa (PEA-15) may block ERK-dependent transcription and proliferation by sequestering ERK in the cytoplasm (Formstecher et al., 2001). Interestingly, PEA-15 contains a NES-sequence and both the mutation of this sequence and exposure to LB causes accumulation of PEA-15 in the nucleus where it still may bind to ERK (Formstecher et al., 2001). Inhibition of NES-dependent transport may thus not only affect nuclear export by MEK, but also by PEA-15 as well as other not yet identified proteins.

Stimulus- and cell type-specific response of ERK

None of the stimuli applied induced nuclear accumulation of pERK in hepatocytes or hepatoma cells, whereas both hypo-osmolarity and EGF led to pERK translocation in a gill cell line. In hepatocytes, this could tentatively be related to their fully differentiated state, as they show no mitotic activity when kept in culture under the conditions applied, as well as to a potential lack of anchorage-dependent structures required for ERK nuclear signalling. However, nuclear translocation also appeared to be absent in well-proliferating and well-adhering trout hepatoma cells, even when NES-dependent export was

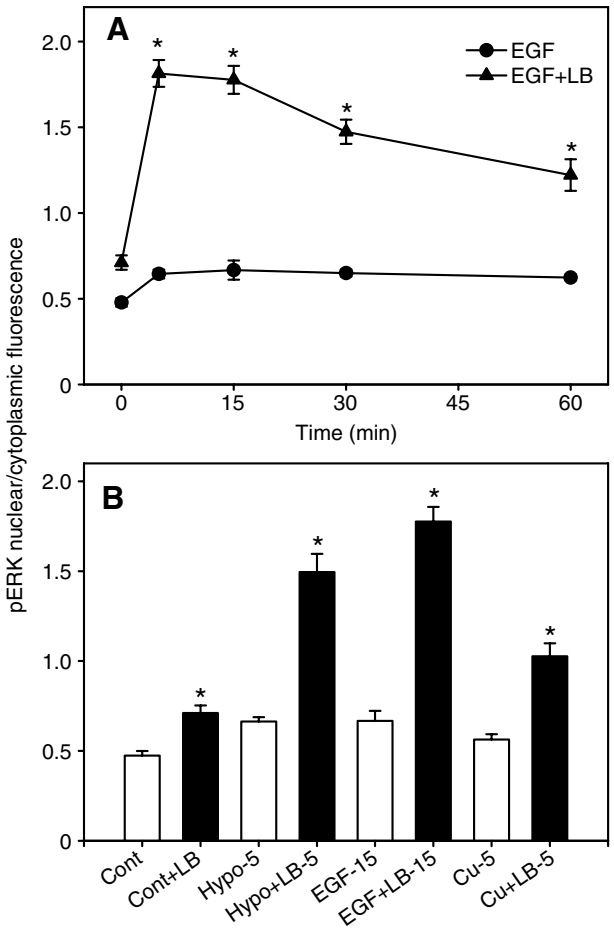

Fig. 4. Quantitative estimation of nuclear translocation of phosphorylated ERK in RTgill-W1 cells, as determined from the ratio of staining intensity measured in the nucleus to that of a cytoplasmic region of the cells. (A) Time course of the change in nuclear/cytoplasmic ratio following the addition of EGF is shown in controls and in cells after pre-incubation with leptomycin B. Values are means ± s.e.m. of at least 20 cells from 2–3 independent cultures. (B) Nuclear/cytoplasmic fluorescence ratio of cells after exposure to hypo-osmotic shock, EGF or Cu at the time where the maximum increase of relative nuclear fluorescence was determined (5′ or 15′). Values are means ± s.e.m. of at least 16 cells from 2–3 independent cultures. Asterisks indicate a statistical significant difference (P<0.05) from the value at time zero (A) or from cells in the absence of LB (B).

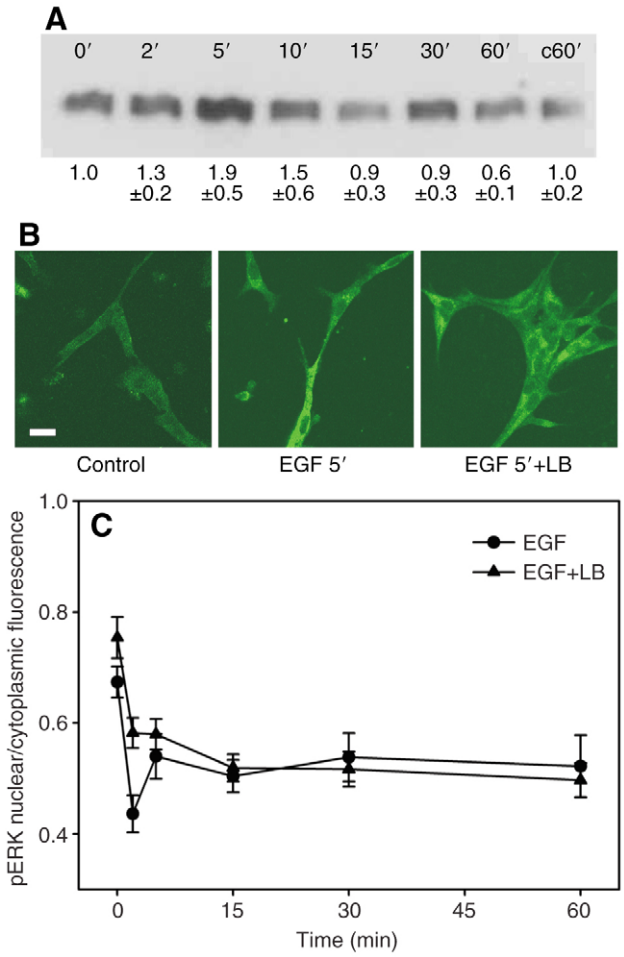

Fig. 5. (A) Dynamics of MEK phosphorylation, determined by western blotting, in RTgill-W1 cells exposed to 30 nmol l^{-1} EGF for up to 60 min. Figures below the blot indicate changes in pMEK abundance, expressed relative to controls at time zero, and denote means ± s.e.m. of four independent experiments. c60, controls incubated in standard conditions for 60 min. (B) Cellular distribution of pMEK in RTgill-W1 cells incubated in standard conditions or exposed to EGF for 5 min. The right image shows cells pre-incubated with leptomycin B (LB) for 60 min prior to addition of EGF. Cells were stained with an antibody against the dually phosphorylated form of MEK and a FITC-labelled secondary antibody and were then examined by confocal laser scanning microscopy. Scale bar, 20 μm (valid for all three images). (C) Quantitative estimation of nuclear/cytoplasmic distribution of phosphorylated MEK, expressed as nuclear/cytoplasmic ratio, in RTgill-W1 cells exposed to EGF with or without prior pre-incubation with LB. Values are means ± s.e.m. of at least 14 cells from three independent cultures. For both treatments, nuclear/cytoplasmic ratio is significantly decreased at all time points compared to the value at time zero (P<0.05).

inhibited by LB. Furthermore, in the case of EGF-stimulation, rather prolonged ERK activation was required to induce cell proliferation in primary rat hepatocytes (Thoresen et al., 2003). Thus, if the same was true for trout hepatocytes or hepatoma cells, nuclear entry of ERK may not be an immediate event in this condition and could have occurred at a later time point not examined here. Similarly, transcriptional activity elicited by hypo-osmolarity may only be induced during prolonged aniso-osmotic exposure of liver cells, and in the short term ERK signalling may be more important for the control of volume regulatory processes. Our earlier study reported that inhibition of MEK was found to delay volume recovery in trout hepatocytes (Ebner et al., 2006), and this is also supported by preliminary experiments on RTH-149 cells (G.K., unpublished observation) and by similar findings on renal epithelial A6 cells exposed to hypo-osmotic conditions (Chiri et al., 2004).

The observation of nuclear accumulation in the gill cell line

might thus mirror cell type specificity of the ERK response. However, before this may actually be linked to functional differences between liver and gill cells, additional studies using primary gill cells are required, as the permanent cell line may not be fully representative of the gill epithelium *in vivo*.

Finally, no evidence of nuclear entry of pERK was found in any cell type in response to Cu exposure. At the concentration applied, Cu is toxic to trout hepatocytes (Krumschnabel et al., 2005) as well as to RTH-149 cells and RTgill-W1 cells in the long term (P. Traunfellner and G.K., unpublished observations). Nevertheless, we saw that Cu caused an increase of pERK levels in hepatocytes, whereas in the cell lines pERK was slightly diminished (RTgill-W1 cells) or unaltered (RTH-149). Generally, ERK is believed to play a dual role during toxic insults, either promoting cell survival (Czaja et al., 2003; Rosseland et al., 2005) or favouring cell death (Chu et al., 2004; Park et al., 2005; Seo et al., 2001; Wang et al., 2000). A recent study on trout hepatocytes suggests that enhanced ERK activity is detrimental during Cu^{2+} exposure (Nawaz et al., 2006). If this was due to the nuclear action of MAP kinase, the absence of nuclear accumulation of ERK could be beneficial for the cells. In line with this, in mouse hippocampal cells it was found that nuclear retention of ERK caused by toxic stimuli promoted cell death (Stanciu and DeFranco, 2002). Similarly, in hydrogen peroxide-exposed rat hepatocytes, ERK, which promoted cell survival, remained cytoplasmic and there it activated the pro-survival ribosomal 6 kinase, which then translocated to the nucleus (Rosseland et al., 2005). Furthermore, several studies have shown that ERK retention in the cytosol induces growth arrest (Brunet et al., 1999; Formstecher et al., 2001; Marenda et al., 2006; Smith et al., 2004), which would seem appropriate in the face of stressful conditions possibly leading to DNA damage.

List of abbreviations

BSA	bovine serum albumin
DTT	dithiothreitol
EGF	epidermal growth factor
ERK	extracellular signal regulated protein kinase
FBS	fetal bovine serum
HRP	horseradish peroxidase
LB	leptomycin B
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
NES	nuclear-export signal
pERK	phosphorylated ERK
PLL	poly-L-lysine
pMEK	phosphorylated MEK
RACE	rapid amplification of cDNA ends
tERK	total ERK

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