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Activation of extracellular signal-regulated kinases during dehydration in the African clawed frog, *Xenopus laevis*

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SUMMARY

In its native environment the African clawed frog, *Xenopus laevis*, can experience seasonally arid conditions that impose dehydration stress. Activation of intracellular signal transduction cascades can mediate and coordinate biochemical responses to ameliorate dehydration stress. This study examines the extracellular signal-regulated kinase (ERK) signaling cascade, analyzing responses of both upstream and downstream components in six tissues of *X. laevis* experiencing medium and high levels of dehydration, 16.6±1.59 and 28.0±1.6% of total body water lost, respectively. Immunoblotting was used to assess the three tiers in this mitogen-activated protein kinase (MAPK) cascade: the initiating MAPK kinase kinases (c-Raf, MEKK), the MAPK kinase (MEK1/2), and finally the MAPK (ERK1/2). The amount of active phosphorylated c-Raf^{Ser338} rose by 2- to 2.5-fold under high dehydration in muscle, lung and skin whereas MEKK protein levels rose in these organs and also increased 4-fold in liver. As a result, phosphorylated active MEK1/2$^{Ser217/221}$ increased significantly by 2- to 6-fold during dehydration which, in turn, led to 2- to 6-fold increases in phospho-ERK$^{Thr202/Tyr204}$ content in all tissues except skin. Given this clear demonstration of ERK cascade activation, two downstream targets of ERK2 were then evaluated. The amount of phosphorylated active transcription factor, STAT3^{Ser727} and p90 ribosomal S6 kinase (RSK^{Ser380}) increased particularly in muscle, lung and kidney. Furthermore, RSK activation was correlated with a 5- to 8-fold increase in phosphorylation of its target, S6 ribosomal protein. Overall, the results show a strong conserved activation of the ERK cascade in *X. laevis* tissues in response to dehydration.

Key words: signal transduction cascade, mitogen-activated protein kinase, signal transducers and activators of transcription, p90 ribosomal S6 kinase, S6 ribosomal protein, dehydration tolerance.

INTRODUCTION

The African clawed frog, *Xenopus laevis*, is native to southern Africa where it lives in swamps, lakes and rivers as well as man-made bodies of water (Tinsley and Kobel, 1996). The frog is largely aquatic and rarely ventures onto land. However, seasonal changes in water levels can make it hard for the frogs to survive through the dry season and they have options including overland migration to new ponds or digging into the mud of evaporating ponds and entering a period of aerobic torpor known as estivation (Tinsley and Kobel, 1996). Both of these can place the frogs under desiccation stress. *Xenopus* sp. have been widely studied since the 1950s as laboratory model animals, most extensively as a model of embryonic development (Beck and Slack, 2001). However, less is known about the responses of this species to environmental stresses, although some studies have looked at nitrogen metabolism and changes in the activities of urea cycle enzymes during dehydration and estivation (Balinsky et al., 1961; Balinsky et al., 1967). The molecular signaling mechanisms that allow adult *Xenopus* to deal with environmental dehydration stress have not been explored. The present study investigated one of the signal transduction cascades that mediate external stress signals to coordinate metabolic and gene expression responses by cells. This is the extracellular signal-regulated kinase (ERK) cascade, one family of the mitogen-activated protein kinase (MAPK) superfamily.

The MAPK superfamily encompasses major conserved signaling cascades that lead to the control of diverse physiological processes (Roux and Blenis, 2004; Krishna and Narang, 2008). MAPKs have been classified into six distinct pathways in mammals based on their preferential response to selected signals (Krishna and Narang, 2008). The three most studied groups of vertebrate MAPKs are: (1) the p38 MAPKs, which were originally described as a 38 kDa protein that is phosphorylated under endotoxin treatment or osmotic shock; (2) the stress-activated protein kinases (SAPKs), also known as JNKs, because they were first discovered though their role in the phosphorylation of the c-Jun transcription factor, and (3) the ERKs (Cowan and Storey, 2003; Krishna and Narang, 2008). All MAPK cascades begin at the cell membrane where an external signal causes the activation of a receptor kinase, typically a receptor protein tyrosine kinase (RPTK). This then triggers a three-tier regulatory cascade of kinases that provide a huge amplification of signal through the serial activation of the MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and finally the MAPK.

The regulation of the three main MAPK members in response to different stresses has been well characterized in mammalian cell lines but until recently the role of this superfamily in regulating adaptive responses in stress-tolerant animals has received little attention (Cowan and Storey, 2003). Recent work in our lab has examined the responses of the three MAPK families to whole body freezing in wood frogs, anoxia exposure in turtles and snails, and hibernation in ground squirrels (Greenway and Storey, 2000a; Greenway and Storey, 2000b; Cowan and Storey, 2003; MacDonald and Storey, 2005; Larade and Storey, 2006). In wood frogs (*Rana sylvatica*), for example, natural freezing survival was associated with the activation of p38 and JNK in different tissues, whereas the ERK pathway was unaffected (Greenway and Storey, 2000a). Studies have also explored the activation of MAPK family members in another

frog species (*Rana ridibunda*) by diverse stimuli, including oxidative stress (Aggeli et al., 2001; Gaitanaki et al., 2003). In the present study we hypothesized that the ERK2 pathway would be activated in response to dehydration stress leading to subsequent activation of downstream ERK targets. Among the targets of ERK are various transcription factors and protein kinases that could play roles in cellular responses to dehydration, including enhanced expression of genes encoding protective proteins as well as protein kinase-mediated readjustment and/or suppression of energy-expensive cellular processes under stress.

This study examines the effect of dehydration on the expression of the ERK2 pathway in *X. laevis* including the upstream regulators of ERK2 and some of its downstream targets. The data show a strong coordinated activation of the entire cascade, providing good evidence of a significant role for ERK signaling in regulating both metabolic and gene expression responses to dehydration in the frogs.

MATERIALS AND METHODS

Animals

Animal holding and experimental procedures received prior approval of the Carleton University Animal Care Committee. Female *Xenopus laevis* (Daudin), 80–128 g body mass, were a donation from the Department of Zoology, University of Toronto. Animals were held in tanks of dechlorinated water at 22°C. Dehydration experiments were similar to those described previously (Churchill and Storey, 1993). Animals were divided into three groups: (1) controls maintained as above, (2) medium dehydration and (3) high dehydration. For medium dehydration, frogs were placed in closed containers and were allowed to air dry over time. For high dehydration, frogs were placed in the same types of containers but these had a layer of silica gel desiccant in the bottom, which resulted in a more rapid rate of dehydration; a perforated divider prevented frogs from touching the desiccant. Water loss was monitored at intervals over 6–7 days by removing the animals at intervals and quickly weighing them. Six frogs were monitored at frequent intervals (Fig. 1) and the others less often. The change in mass was used to calculate the percentage of total body water lost using the following equation:

$$\%\ \text{water lost} = [(W_\text{i} - W_\text{d}) / (W_\text{i} \times BWC_\text{i})] \times 100\ ,$$

where W_i is the initial mass of an individual, W_d is the mass during experimental dehydration, and BWC_i is the initial body water content of frogs before dehydration. BWC_i was determined from measurements of the wet and dry masses of seven killed frogs; the value was $0.741\pm0.019\ \text{g}\ H_2O\ \text{g}^{-1}$ body mass. Medium and high dehydration groups of frogs were sampled when they reached predetermined levels of water loss. For tissue sampling, frogs were quickly pithed, blood samples were taken (see below), and then tissues were quickly dissected out and frozen in liquid nitrogen before transfer to –80°C for long-term storage.

Blood sampling, hematocrit and metabolic assays

Blood was sampled as follows. The aorta was snipped and two 50 µl aliquots of blood were quickly withdrawn into heparinized micropipettes. These were centrifuged for 1 min and then total sample length and packed red blood cell length in the tubes were measured and used to calculate the hematocrit. Remaining blood was pipetted into 2 ml microcentrifuge tubes with an aliquot (~0.1 vol.) of anticoagulant solution added [10 mmol l^{-1} EDTA in 0.9% (w/v) NaCl]. Samples were centrifuged for 5 min at 5°C and then plasma was removed and frozen at –80°C. Plasma glucose levels were measured using a commercial glucose analyzer (Freestyle, Alameda, CA, USA). Initial tests with known amounts of glucose added to aliquots of *X. laevis* plasma showed that there were no detection problems when using frog plasma with this commercial analyzer.

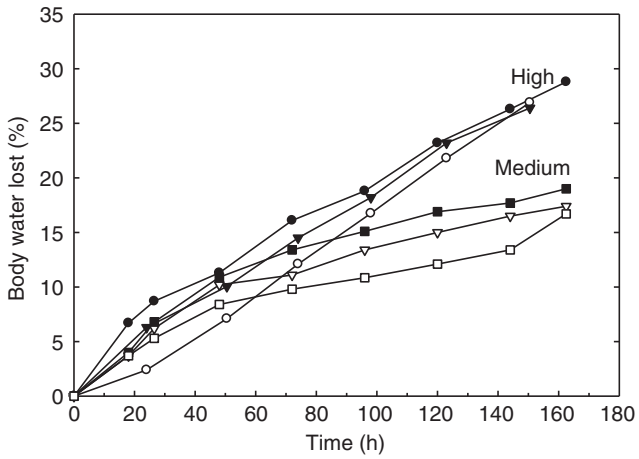

Fig. 1. Progress of body water loss by six *X. laevis* over 6–7 days of air drying (medium dehydration) compared with drying over desiccant (high dehydration); conditions are described in detail in the Materials and methods. For the animals shown, final percentage body water loss values were 16.7, 17.4 and 19% for medium and 26.4, 26.9 and 28.8% for high dehydration treatment.

Plasma urea was measured by a colorimetric assay (Price and Harrison, 1987) with some modification. The working reagent was made fresh by combining 2 vol. stock reagent A [600 ml double-distilled water (DDW), 300 ml H_2SO_4, 100 ml 85% H_3PO_4, 0.1 g $FeCl_3$] with 1 vol. stock reagent B (500 ml DDW, 0.05 g thiosemicarbazide, 2.5 g diacetylmonoxime). Samples (1–50 µl plasma) and standards (from 1 mmol l^{-1} urea stock) were added to polypropylene Falcon tubes and adjusted to 1 ml total volume with DWW. A 0.5 ml aliquot of the working reagent was then added, mixed and the tubes were boiled in a covered bath for 10 min. After cooling for a few minutes in a fume hood, 200 µl aliquots of samples were pipetted into wells of a microplate and absorbance at 525 nm was measured using a microplate reader. Urea concentrations in plasma samples were determined by comparison with the standard curve.

Preparation of crude tissue extracts

Frozen tissue samples were homogenized 1:10 (w/v) in buffer containing 20 mmol l^{-1} Hepes, 0.1 mmol l^{-1} EDTA, 1 mmol l^{-1} Na_3VO_4, 200 mmol l^{-1} NaCl, 10 mmol l^{-1} NaF, 10 mmol l^{-1} β-glycerophosphate, and Sigma protease inhibitor mix. After centrifugation for 15 min at 24,000 ***g***, the supernatant was removed. Soluble protein concentration was measured by the Coomassie Blue dye-binding method using Bio-Rad (Hercules, Ca, USA)-prepared reagent and bovine serum albumin as the standard. Samples were adjusted (if needed) to constant concentrations by the addition of small amounts of buffer and were then mixed 1:1 (v/v) with SDS-PAGE sample buffer [100 mmol l^{-1} Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.2 (w/v) Bromophenol Blue, 10% (v/v) 2-mercaptoethanol] and boiled for 5 min. Final soluble protein concentrations for the preparations from different tissues were 5 µg µl^{-1} for heart and kidney, 4 µg µl^{-1} for skeletal muscle, 2 µg µl^{-1} for lung and liver and 1 µg µl^{-1} for skin.

Immunoblotting

Aliquots containing equal amounts of protein (10–30 μg depending on the protein being analyzed) were loaded into wells of 10 or 12% SDS polyacrylamide gels. Electrophoresis was carried out on a Bio-Rad mini-gel apparatus at room temperature using 1× running buffer (3.02 g Tris base, 18.8 g glycine, 1 g SDS l^{-1}, pH~8.3). Proteins were then transferred onto PVDF membrane by wet transfer with transfer buffer containing 25 mmol l^{-1} Tris pH 8.5, 192 mmol l^{-1} glycine, 20% (v/v) methanol. Membranes were washed in TBST [Tris-buffered saline containing 0.1% (v/v) Tween 20, 20 mmol l^{-1} Tris base, 140 mmol l^{-1} NaCl] for 5 min and were then blocked with 2.5% non-fat dried milk for 10–30 min in TBST before washing twice with TBST. Membranes were then incubated with primary antibodies overnight at 4°C followed by washing for 3× 5 min in TBST and then incubation with secondary antibody (1:1000 or 1:2000 dilution) for 1–2 h at 22°C, and final washes of 3× 5 min with TBST. Immunoreactive bands were visualized by enhanced chemiluminescence (Millipore Corporation, Billerica, MA, USA). PVDF membranes were then stained for 20 min with Coomassie Blue [0.25% (w/v) Coomassie Brilliant Blue R, 50% (v/v) methanol, 7.5% (v/v) acetic acid] and destained for ~7 min with destaining solution (50 ml distilled water, 50 ml acetic acid, 150 ml methanol). Antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) except for MEK kinase and MEK1/2, which were from Transduction Laboratories (Lexington, KY, USA); all were used according to manufacturer's instructions.

Normalization and statistics

Both immunoblot and Coomassie-stained bands were visualized using a ChemiGenius Bio-imaging System (Syngene, Frederick, MD, USA) and densitometric analysis was performed using the associated Gene Tools software. A group of three Coomassie-Blue-stained bands that showed constant density in all samples from a given tissue were quantified and immunoblot band densities in each lane were normalized against the summed density of these bands in the same lane in order to correct for any minor variations in sample loading. Mean normalized band densities ± s.e.m. for control and dehydrated samples (N=3–6 independent samples) were calculated and then statistical testing was performed using analysis of variance followed by the Student–Newman–Keuls test.

RESULTS AND DISCUSSION

Metabolic responses to dehydration

Initial studies determined the limit of body water loss for *X. laevis*; frogs died at values just above 30%, the mean percentage of total body water loss for frogs that died being 32.4% ±0.98 s.e.m. (N=7). Based on this value, two groups of frogs were set up and monitored as they underwent experimental dehydration to medium or high levels. Frogs lost water at near-linear rates and the final mean percentages for the medium and high dehydration groups were 16.6±1.6% (N=9) and 28.0±1.6% (N=10), respectively, of total body water lost over 6–7 days (Fig. 1). Hence, the high dehydration group represents frogs whose water loss was close to the lethal limit whereas the medium dehydration group reached only about half of that level. Blood hematocrit values also attested to the loss of body water, the percentage of total blood volume occupied by packed red blood cells rising from 40% in control frogs to 47% in medium and 50% in high dehydration groups (Table 1).

The high cutaneous water loss rates of amphibians can be minimized by behavioral, physiological and biochemical adaptations (Hillman et al., 2009). One method to help retard water loss is to raise the osmolality of body fluids and, among anuran species that estivate or endure hypersalinity, the commonly occurring osmolyte is urea. Indeed, previous studies have shown that under osmotic or water stress, *X. laevis* reduce ammonia excretion, elevate the activities of urea cycle enzymes and accumulate urea (Balinsky et al., 1961; Janssens, 1964; Seiter et al., 1978). In the present study, plasma urea concentration increased sharply when frogs were dehydrated, rising by 27- and 33-fold, respectively, in the medium and high dehydration groups as compared with control values of 1.66 mmol l^{-1} (Table 1). As a consequence of dehydration, amphibians also experience hypoxia as a result of elevated blood viscosity and reduced blood volume that make it harder to fully oxygenate tissues (Hillman et al., 2009). Hypoxia stress typically triggers the mobilization of liver glycogen reserves and elevates plasma glucose in frog species, and in the present study this also occurred. Plasma glucose levels in the high dehydration group were 2.3-fold higher than in controls (Table 1), similar to the effects of dehydration on blood glucose levels in leopard frogs, *Rana pipiens* (Churchill and Storey, 1993). This is consistent with the probable development of hypoxic conditions during dehydration in *X. laevis*.

The ERK pathway

The components of the ERK signaling cascade have been well established (Roux and Blenis, 2004; May and Hill, 2008; Ramos, 2008). The classical initiation site is a RPTK in the plasma membrane. Activation of the receptor passes a signal from its phosphotyrosine-docking protein and adaptor growth factor receptor bound proteins (Grb2) to a guanine nucleotide exchange factor called SOS (son of sevenless). SOS facilitates the activation of a small G protein called Ras by catalyzing the exchange of GDP to GTP. Active, GTP-bound Ras then binds to and activates one of the MAPKKKs belonging to the ERK cascade; these include Rafs (A-Raf, B-Raf, and c-Raf), Mos and MEKK. These then activate the MAPKKs (MAPK/ERK kinase or MEK1/2) and finally the MAPKs (the ERK family, ERK1 and ERK2 being the best studied) (Fig. 2). The activated ERK is then capable of phosphorylating cytosolic substrates or transcription factors.

Table 1. Total body water lost by experimental groups of *X. laevis*, blood hematocrit and plasma urea and glucose concentrations

	Control	Medium dehydration	High dehydration
Body water lost (%)	0	16.6±1.6	28.0±1.6‡
Hematocrit (%)	40.4±1.61	46.7±1.13*	49.6±2.24†
Plasma urea (mmol l^{-1})	1.66±0.29	45.8±9.81†	54.8±6.75†
Plasma glucose (mmol l^{-1})	2.52±0.22	3.39±0.36	5.81±0.75†,‡
N	6–13	6–9	6–10

Data are means ± s.e.m. Statistical testing used analysis of variance followed by the Student–Newman–Keuls test. *,†Significantly different from the control value, *P<0.05; †P<0.005; ‡significantly different from the value for medium dehydration, P<0.005.

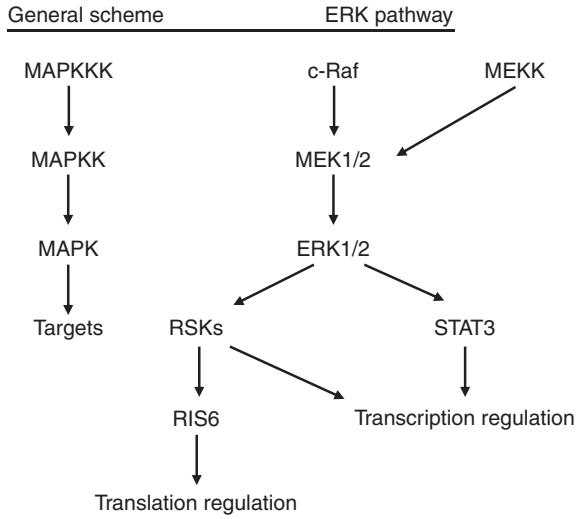

Fig. 2. Schematic of the ERK pathway members analyzed and their relationship to the general MAPK signaling cascade. Arrows lead from activators to target proteins.

Activation of c-Raf and MEKK in response to dehydration

In the present study, immunoblotting was used to measure the levels of phosphorylated c-Raf1 because it has an equal ability to phosphorylate MEK1 and MEK2 (Wu et al., 1996). Different and tissue-specific responses were seen in organs of the medium and high dehydration groups of *X. laevis* (Fig. 3A). Medium dehydration did not significantly affect phospho-c-Raf^{Ser338} levels in any of the five tissues tested but high dehydration did. Phospho-c-Raf^{Ser338} levels rose in skeletal muscle and lung by 2.4- to 2.5-fold (P<0.01) compared with control levels in both organs. Similarly, levels of the phosphoprotein rose by 94% in skin (P<0.05) as compared with control levels. Phospho-c-Raf^{Ser338} was not detectable in the liver.

Another MAPKKK in the ERK pathway is MEKK (Roux and Blenis, 2004) and analysis of MEKK showed that it also responded to dehydration in *X. laevis* tissues. MEKK total protein levels were elevated significantly in muscle and liver of the medium dehydration group of frogs by 55% and 4-fold (P<0.01), respectively (Fig. 3B). Levels remained elevated in liver of the high dehydration group of frogs. MEKK protein also responded to high dehydration in lung and kidney, rising by 2- and 2.3-fold. However, in heart and skin, MEKK levels were reduced significantly in dehydrated frogs and MEKK levels also returned to control values in skeletal muscle of the high dehydration group. The activation of c-Raf and MEKK in the present study is also in line with previous findings (Matsuda et al., 1995) on the effect of osmotic shock on these proteins in rat 3Y1 fibroblastic cells. Our data support the proposal that c-Raf and MEKK both play important roles in the response of *X. laevis* lung and skeletal muscle to dehydration. MEKK is also important in liver and kidney whereas c-Raf responded positively to dehydration in skin.

MEK1/2 activation in response to dehydration

MEKK, c-Raf, and other activated MAPKKKs phosphorylate the dual specificity kinases MEK1 and 2. These are highly selective for their substrates, which are primarily ERK1 and ERK2 (English et al., 1999). They phosphorylate ERK1/2 at both tyrosine and serine/threonine residues (MacDonald and Storey, 2004), and MEK1/2 have two other unique features that allow them to efficiently activate ERK1/2. The first is the binding site of ERK2,

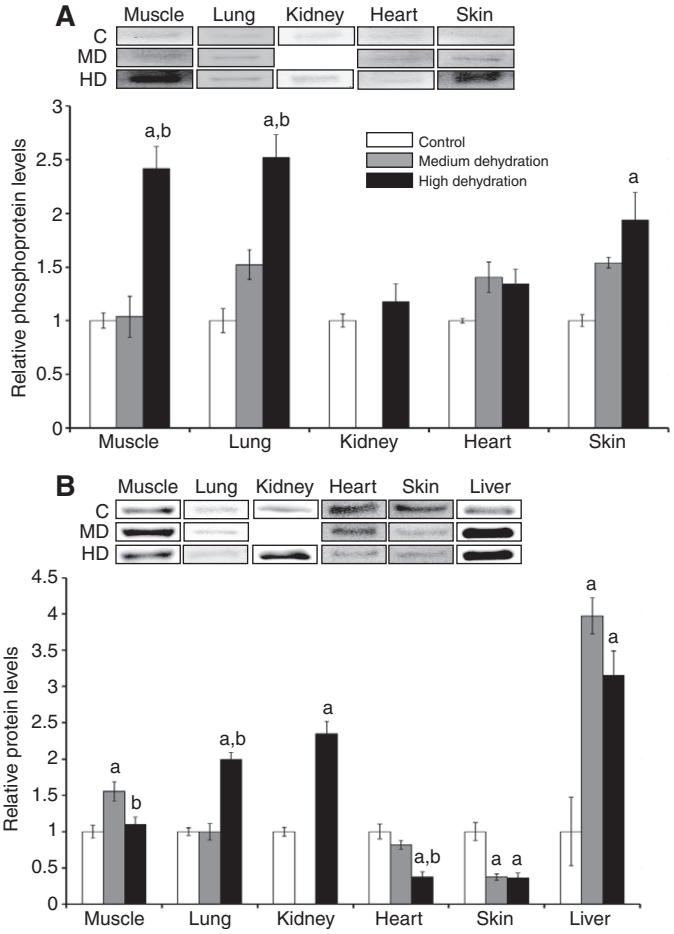

Fig. 3. Effect of medium and high dehydration on the activation of two MAPKKKs, c-Raf and MEKK, in *X. laevis* tissues. (A) Expression levels of active phosphorylated c-Raf. The phospho-c-Raf^{Ser338} antibody crossreacted with a single band at the expected molecular mass, ~74 kDa. (B) MEKK protein levels. The MEKK antibody crossreacted with a single band at the expected molecular mass of ~45 kDa. In A and B, representative western blots show control (C), medium dehydration (MD) and high dehydration (HD) conditions. Histograms show mean normalized protein levels, expressed relative to control values. Kidney samples from frogs given the medium dehydration treatment were not available. Data are means ± s.e.m., N=3-6 independent trials on tissue extracts from different animals. a: significantly different from the corresponding control, P<0.05; b: significantly different from the corresponding medium dehydration value, P<0.05.

which is an eight residue stretch in MEK1/2 known as the D domain. The second feature is the presence of a proline-rich insert in the C-terminal portion of the catalytic core of MEK1/2, which is important for intracellular stimulation of ERK1/2 (English et al., 1999).

Since upstream regulators of MEK1/2 were activated in response to dehydration in some *X. laevis* organs, we next measured total protein levels and activation status of MEK1/2. In skin and liver, total MEK1/2 protein decreased by ~50 and ~60% (P<0.01), respectively, under both medium and high dehydration conditions (Fig. 4A). MEK1/2 levels were also reduced in heart of the medium dehydration group of frogs by 33% (P<0.01). However, in lung, total MEK1/2 protein content was strongly increased by 2- and 4-fold (P<0.01) under medium and high dehydration, and also increased 2-fold (P<0.01) in the kidney under high dehydration.

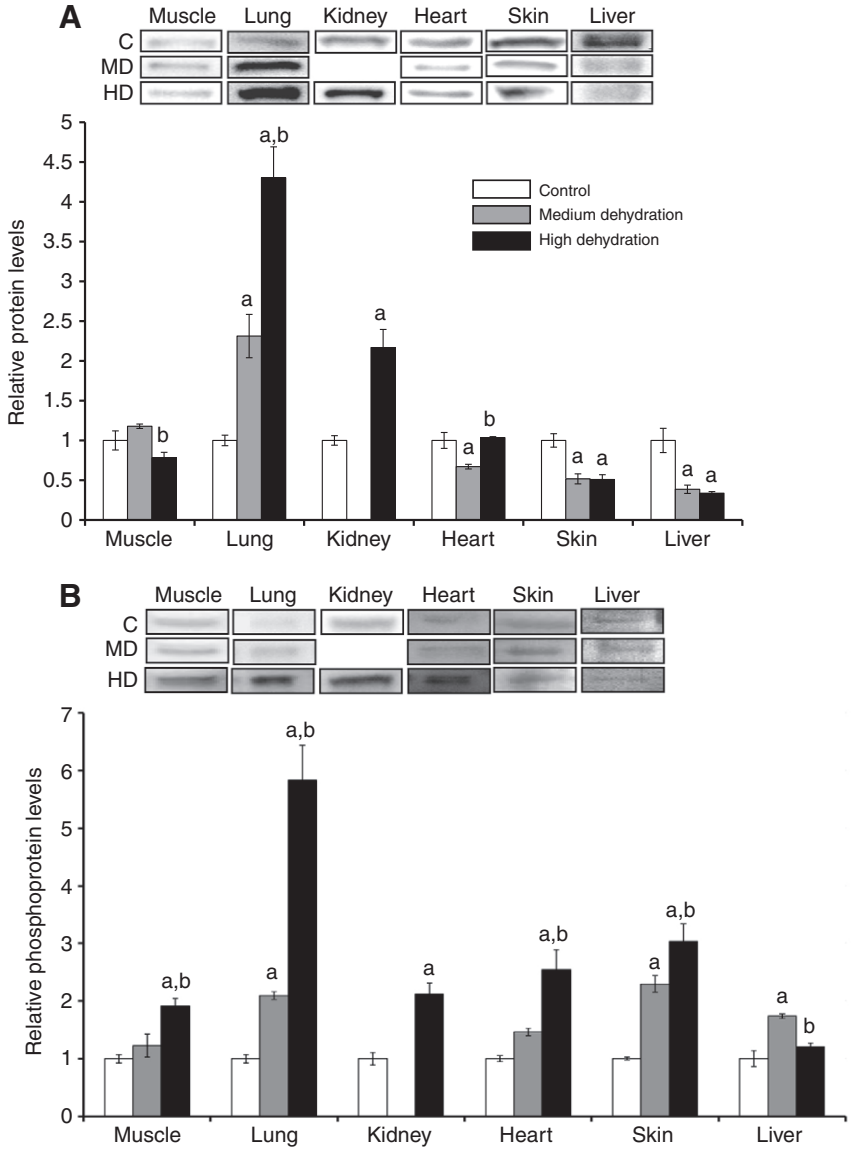

Fig. 4. Effect of medium and high dehydration on the MAPKK of the ERK cascade in six tissues of *X. laevis*. (A) Total MEK1/2 protein levels and (B) phospho-MEK1/2$^{Ser217/221}$ levels. The antibodies used crossreacted with single bands at the expected molecular mass of ~45 kDa. Other information as in Fig. 2.

It is the active phosphorylated form of MEK that is key to triggering ERK and so the relative levels of phospho-MEK1/2$^{Ser217/221}$ are important. Fig. 4B shows that dehydration enhanced phosphorylation of MEK in all tissues, compared with control levels. Under medium dehydration conditions, the amount of phospho-MEK1/2$^{Ser217/221}$ increased by 2-fold in lung, 2.3-fold in skin and by 74% in liver (P<0.01). High dehydration further enhanced phospho-MEK1/2$^{Ser217/221}$ content in lung and skin to 6-fold and 3-fold above the phosphoprotein content of controls (P<0.01). Phospho-MEK1/2$^{Ser217/221}$ levels were also elevated by 1.8-fold in muscle, 2-fold in kidney, and 2.5-fold in heart (P<0.01) under high dehydration conditions.

A strong correlation between c-Raf activation (Fig. 3A) and the phosphorylation of its downstream target MEK1/2 (Fig. 4) occurred under high dehydration in muscle, lung and skin. Similarly, there was a good correlation between MEKK activation (Fig. 3B) and elevated levels of phosphorylated MEK1/2 in kidney, lung and liver. However, the elevated phospho-MEK1/2$^{Ser217/221}$ levels in heart under high dehydration conditions could not be related to either c-Raf or MEKK so it is possible that other Raf isoforms or Mos could be involved in the pathway in heart (Roux and Blenis, 2004; Sridhar et al., 2005).

ERK2 activation in response to dehydration

Activation of MEK1/2, the MAPKK of the ERK pathway, leads to phosphorylation of ERK at a conserved Thr-Glu-Tyr (TEY) motif of the activation loop (MacDonald and Storey, 2004). The vertebrate ERK family includes six isozymes (ERK1, 2, 3, 5, 7, 8) but ERK1 and ERK2 have received the most study. They share 83% amino acid identity and are structurally and functionally similar (Roux and Blenis, 2004). In general, activation of the ERK pathway mediates cell growth and differentiation in response to a number of different stimuli including growth factors, cytokines, transforming agents, virus infection and carcinogens (May and Hill, 2008; Ramos, 2008). The interaction of ERK2 (the isozyme analyzed in the present study) with its substrates was better understood after using X-ray crystallography. The activation of ERK2 causes the protein to undergo conformational changes, creating a proline-specific surface pocket. When inactive, this pocket is usually occupied by Arg192, so substrate access to the proline catalytic site is inhibited when ERK2 is not phosphorylated on Thr183 and Tyr185. In addition to the proline-specific pockets, ERK2 has the ability to regulate its substrates through direct binding of the C-terminal substrate binding groove of ERK to the

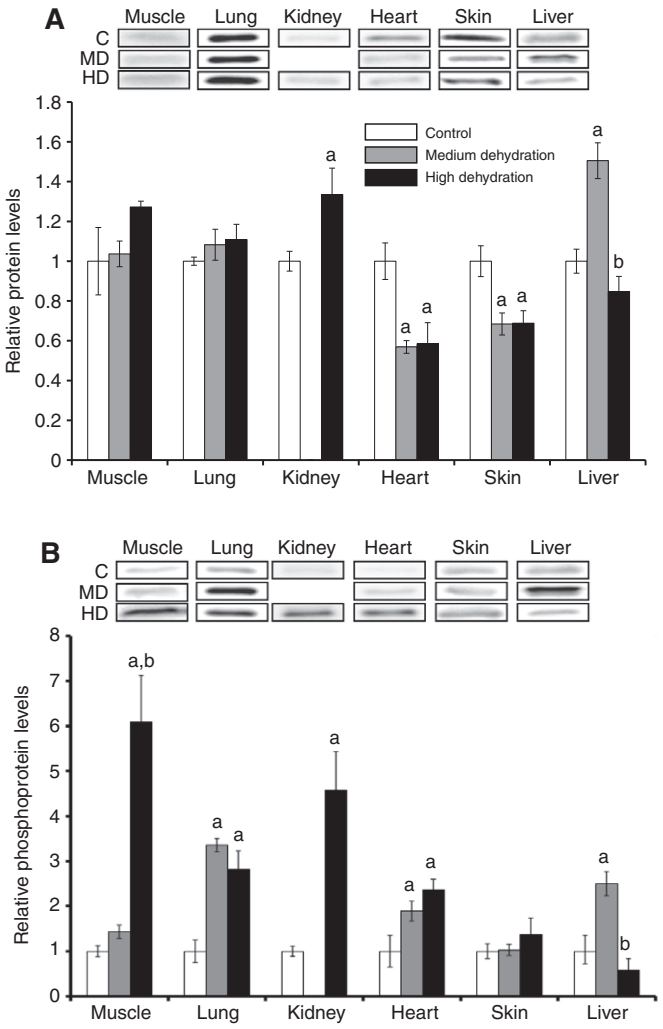

Fig. 5. Effect of medium and high dehydration on (A) total ERK2 protein levels and (B) phospho-ERK$^{Thr202/Tyr204}$ content in tissues of *X. laevis*. The antibody used crossreacted with a single band at the expected ~42 kDa. Other information as in Fig. 2.

activation site of substrates, thereby restricting their interaction with other kinases (Schaeffer and Weber, 1999).

The ERK2 antibody crossreacted with a single band in *X. laevis* tissues at the expected molecular mass for ERK2 (~42 kDa). Under dehydration stress the total protein levels of ERK2 remained stable in muscle and lung of *X. laevis* (Fig. 5A) but ERK2 levels were suppressed by 30–40% (P<0.05) in heart and skin of both the medium and high dehydration group of frogs. By contrast, total ERK2 protein increased by 35 and 50% (P<0.01) in kidney and liver, respectively, under medium dehydration. The active, phosphorylated form of ERK2 showed very different responses to dehydration, with a significant increase in the amount of active phospho-ERK$^{Thr202/Tyr204}$ detected in all tissues except skin (Fig. 5B), mirroring the activation of MEK1/2, the upstream MAPKK (Fig. 4). In particular, phospho-ERK$^{Thr202/Tyr204}$ content was very strongly enhanced in both muscle and kidney, by 6- and 4.6-fold (P<0.01), respectively, under high dehydration conditions as compared with control levels of the phosphoprotein. Phospho-ERK$^{Thr202/Tyr204}$ levels also increased in lung by 2.8- to 3.3-fold (P<0.01) in dehydrated animals, and heart showed a similar approximate 2-fold increase. This result is consistent with previous work on *R. ridibunda* heart in which ERK was found to be powerfully activated by stress stimuli (Gaitanaki et al., 2003). A 2.5-fold increase (P<0.01) in the amount of phosphorylated active ERK2 was also seen in liver during medium dehydration.

Overall, then, the above data for the MAPKKK, MAPKK and MAPK components of the ERK cascade are all consistent in documenting an activation of this cascade in response to physiological dehydration stress in most tissues of *X. laevis.* The exception was skin, which showed elevated levels of phosphorylated c-Raf and MEK1/2 but not of ERK2. However, it is possible that ERK1 might be the target that is activated in skin. Activation of the ERK cascade may be responding directly to dehydration stress or may be responding to hypoxic conditions caused by dehydration effects on the circulatory system. Studies on other species have reported ERK activation by ischemia and/or reperfusion in rabbits (Iliodromitis et al., 2006) or by osmotic stress in animals and yeast (Kultz and Burg, 1998).

ERK downstream substrates

The activation of the ERK cascade in *X. laevis* tissues indicates that activation of some downstream targets of ERK is important for dehydration resistance. ERK substrates are present in all cellular compartments (Roux and Blenis, 2004), but the best-known actions of ERKs are in the regulation of gene expression. ERKs can translocate to the nucleus and influence gene transcription by phosphorylating transcription factors or transcription factor modifying proteins (Krishna and Narang, 2008; Ramos, 2008). Therefore, we next evaluated the responses of some known ERK targets in *X. laevis* tissues.

The signal transducer and activators of transcription (STAT) proteins are a family of transcription factors that form homo- or heterodimers upon phosphorylation before translocating from the cytoplasm into the nucleus. In mammals there are seven family members (STAT 1, 2, 3, 4, 5a, 5b, 6). Phosphorylation of these transcription factors occurs on a conserved tyrosine residue in the C-terminal domain (Benekli et al., 2003; Ning et al., 2001). STAT3 is a known substrate of ERK1/2 (Chung et al., 1997; Krishna and Narang, 2008) and so we analyzed the phosphorylation status of phospho-STAT3^{Ser727} as a measure of ERK downstream action. Levels of the phosphoprotein increased significantly under high dehydration conditions in three tissues of *X. laevis* (Fig. 6A) and in all cases this correlated with elevated phospho-ERK2 in the same tissues (Fig. 5B). In skeletal muscle, phospho-STAT3^{Ser727} showed small but significant increases of 24–30% (P<0.05), whereas greater increases of 1.9- to 2.3-fold (P<0.01) occurred in lung. Phospho-STAT3^{Ser727} levels were also 2-fold higher in kidney of the high dehydration group of frogs (P<0.01). However, phospho-STAT3 levels were suppressed in heart of the medium dehydration group and were unchanged in the other tissues. Activated STAT3 in muscle, lung and kidney can upregulate a number of genes whose protein products assist in cell survival. For example, the anti-apoptotic Bcl2 family members Bcl-x_L and Mcl1 were upregulated in multiple myeloma following STAT3 induction. Other known STAT3 target genes include c-Myc, which plays a role in sensitizing cells to apoptotic stimuli (Benekli et al., 2003). The activation of STAT3 in the muscle and kidney could be a general response to dehydration stress since a significant change in the water contents of individual organs occurs (measured as a change in protein concentration) when frogs dehydrate (Churchill and Storey, 1993; Holden and Storey, 1997).

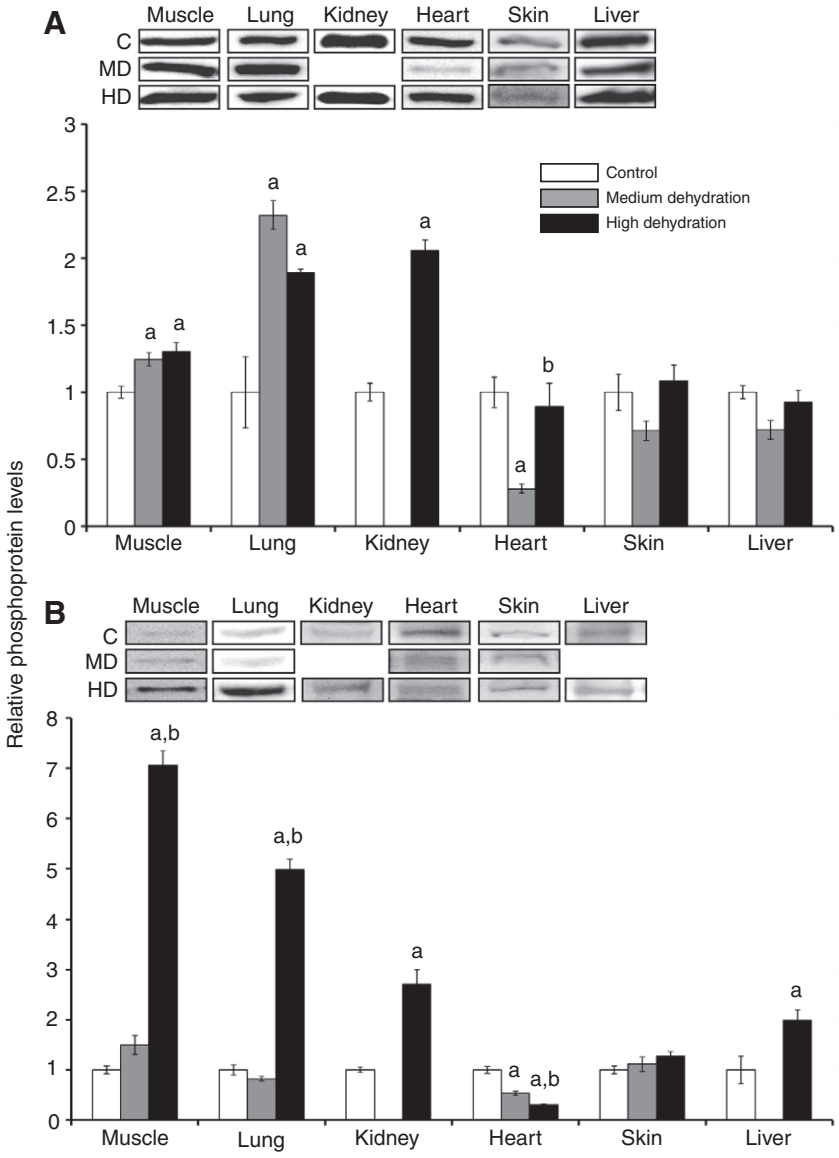

Fig. 6. Effect of medium and high dehydration on the phosphorylation of two target substrates of ERK: (A) phospho-STAT3^{Ser727} and (B) phospho-p90 RSK^{Ser380} in *X. laevis* tissues. Phospho-specific antibodies each crossreacted with single bands on the blots at the expected molecular masses of ~78 kDa for phospho-STAT3^{Ser727} and ~90 kDa for phospho-p90 RSK^{Ser380}. Other information as in Fig. 2.

The p90 ribosomal S6 kinase (RSK) family of proteins is another known target of ERK action. The protein was originally isolated from *Xenopus*, and described as a protein kinase with the ability to phosphorylate the S6 protein of the 40S ribosomal subunit. Although S6 is still the best-known RSK target, recent studies have identified other targets including other proteins involved in ribosomal translation, the insulin receptor, the proapoptotic protein BAD and some nuclear targets (Ruvinsky and Meyuhas, 2006). In humans, there are four RSK isoforms; each contains two distinct and functional kinase domains. In the carboxy-terminal domain, RSK autophosphorylation and activation occur and then stimulate the phosphotransferase activity of the amino-terminal kinase domain to allow RSK to phosphorylate its targets. ERK activation of RSK1 requires the phosphorylation of its C-terminal activation loop (Anjum and Blenis, 2008). ERK achieves that by playing one of two roles in the process. Activated ERK can directly phosphorylate RSK1 and ERK can also bring RSK1 closer to membrane-associated kinases that may phosphorylate RSK on specific residues (Richards et al., 2001).

The present study focused on the regulation of one of the essential RSK proteins, RSK1. The RSK1 activation pattern quite closely mirrored the pattern seen for ERK$^{Thr202/Tyr204}$. High dehydration elevated the amount of p90^{RSKSer380}, with levels of the phosphoprotein increasing by 7-, 5-, 3- and 2-fold (P<0.01) in muscle, lung, kidney and liver, respectively (Fig. 6B). By contrast, in heart, phosphoprotein content decreased by 47% and 69% (P<0.01) during medium and high dehydration, respectively, where the phospho-RSK1 content did not change in skin.

To confirm that RSK activation leads to phosphorylation of downstream targets in tissues of dehydrated frogs, we next measured S6 ribosomal protein (S6RP) levels and its phosphorylation state in *X. laevis* organs. Phosphorylation sites on S6RP were mapped to five serine residues in the highly conserved carboxyl terminus: Ser235, Ser236, Ser240, Ser244 and Ser 247 (Ruvinsky and Meyuhas, 2006). The effects of dehydration on total S6RP protein levels in *X. laevis* tissues are shown in Fig. 7A (note that selected samples were unavailable in three cases). S6 protein levels were 6.9- and 2.4-fold higher (P<0.01) in muscle and liver during high dehydration, as compared with controls. Dehydration also had a very marked effect on lung S6RP protein levels, with mean increases of 23.5- and 17.8-fold (P<0.05) under medium and high dehydration conditions, respectively. However, dehydration had no significant

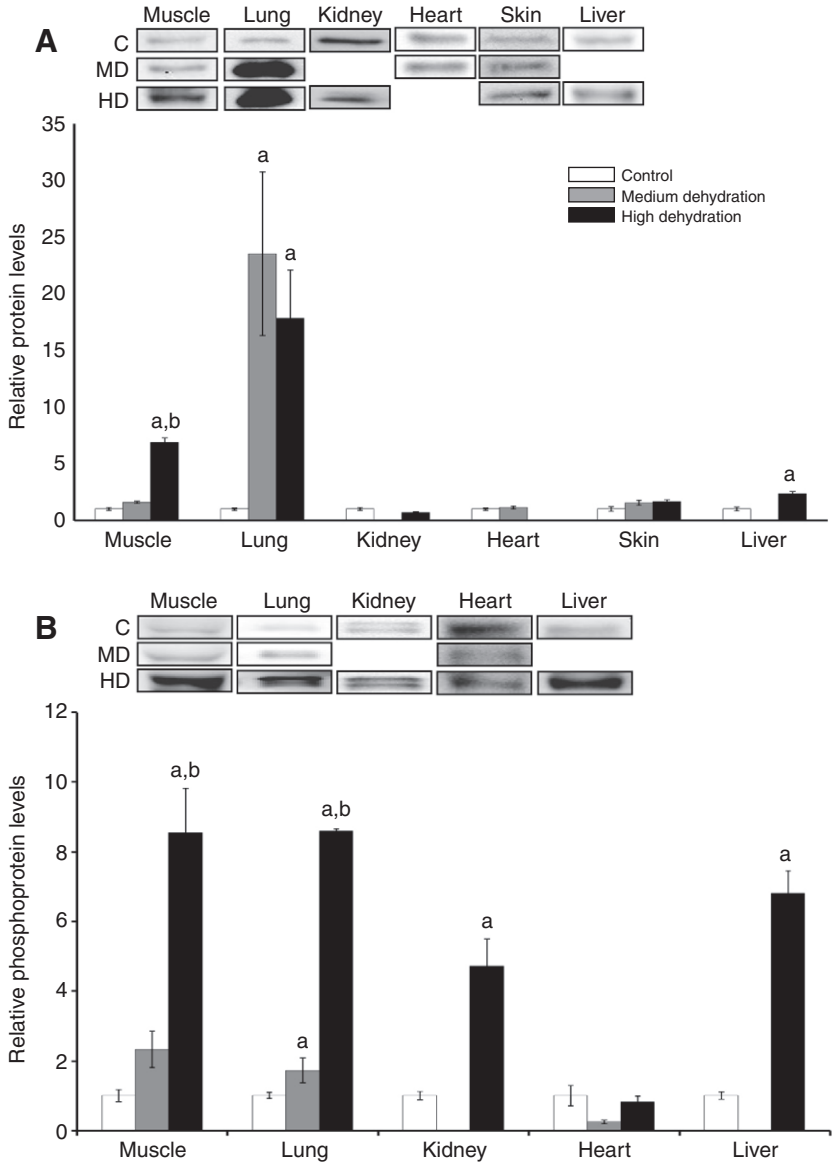

Fig. 7. Effect of medium and high dehydration on (A) total S6 ribosomal protein and (B) phospho-S6 ribosomal$^{Ser235/236}$ protein levels in six tissues of *X. laevis*. S6 antibodies crossreacted with a single band at the expected molecular mass of ~32 kDa. Other information as in Fig. 2.

effect on S6RP protein content in kidney, heart or skin. The phosphorylated form of the protein was assessed using an antibody that detects serine 235/236 phosphorylation in the C-terminal region. A strong increase in phospho-S6RP content was detected in most tissues under high dehydration conditions (Fig. 7B); levels were 8.5-, 8.5-, 4.7- and 6.8-fold higher in skeletal muscle, lung, kidney and liver, respectively (P<0.01), compared with the control tissues. Phospho-S6RP$^{Ser235/236}$ also increased in lung by 70% (P<0.05) under medium dehydration. A good correlation occurred between the increase in phospho-S6RP protein levels and the amount of activated phospho-RSK1 (Fig. 6B) in muscle, lung, kidney and liver of the high dehydration frogs. These findings also agree with previous studies that showed that RSKs are the kinases responsible for phosphorylating the S6RP subunit in *X. laevis* oocytes (Schwab et al., 1999).

The functional role of S6RP phosphorylation is still debatable. Initial studies proposed that S6RP has a regulatory role in translation initiation but subsequent data was conflicting as to whether a causal link existed between S6RP phosphorylation and the efficiency of protein synthesis (Ruvinsky and Meyuhas, 2006). Indeed, a recent study used S6RP knockin (*S6RP*$^{p-/-}$) mice to assess S6RP function with results that showed that the rate of global protein synthesis in mouse embryo fibroblasts was actually higher than in wild-type cells, and, furthermore, the liver of these mice had the same proportion of ribosomes engaged in polysomes as did the wild-type liver. Other actions for phosphorylated S6RP have been suggested including: (1) specifically increasing the translation efficiency of a subgroup of mRNA species that contain 5′-terminal oligopyrimidine tracts (TOP mRNAs), (2) mediating an inverse regulation of cell size *versus* cell proliferation, and (3) involvement in glucose homeostasis. Given the profound changes in S6RP levels and phosphorylation status in *X. laevis* organs, the effects of dehydration stress in this frog model might be an excellent one to examine in order to finally identify a physiological role for S6RP.

Conclusions

As hypothesized, the present study clearly shows that the ERK2 signal transduction cascade is activated in *X. laevis* tissues in response to dehydration. Activation was particularly pronounced under high dehydration conditions, whereas fewer and less pronounced effects were seen in response to medium dehydration. This suggests that responses regulated by ERKs may be defensive

actions to deal with severe dehydration and some of its consequences such as hypoxia caused by elevated blood viscosity. Given that all anurans have wide tolerances to changes in body hydration and osmolality, it appears probable that the stress represented by medium dehydration could be dealt with largely without the intervention of ERK-mediated responses. Activation of the ERK cascade was particularly apparent in lung, which is an organ that is highly susceptible to water loss during breathing. Perhaps ERK signaling might trigger early protective measures by lung to help limit respiratory water loss. In this regard, it is interesting to note that the expression of mucin genes in the mammalian respiratory tract is under ERK1/2 control (Choi et al., 2009), which could suggest that ERK-mediated changes to the amount or composition of mucins in the airway epithelia of *X. laevis* could be involved in limiting respiratory water loss under desiccating conditions. This remains to be experimentally tested. All organs showed activation of some or all of the components of the ERK2 cascade (c-Raf, MEKK, MEK1/2, ERK1/2) in response to high dehydration, which shows that ERK signaling is important to cellular defense when water loss becomes severe. Two well-known downstream targets, STAT3 and RSK1, were also frequently phosphorylated and activated in *X. laevis* tissues in response to dehydration, as was the RSK target, the S6 ribosomal protein. This suggests that these targets may contribute to dehydration defense. Some organs (e.g. heart) showed clear activation of the ERK cascade but no change in the downstream targets that were assessed. However, there are a large number of downstream targets of ERK2, so this suggests that dehydration-stimulated ERK signaling is focused on alternative targets in heart. Skin showed a strong increase in c-Raf and phospho-MEK1/2 but not in other cascade components, which suggests that skin responses to dehydration are not directed by ERK2 but are probably accomplished by another ERK member, such as ERK1. Overall, the study provides strong evidence of a role for ERK signaling in dehydration defense in *X. laevis* organs, documents activation or upregulation of well-known ERK targets, and directs our attention to detailed studies of ERK-mediated responses to uncover all of the metabolic adaptations that are coordinated by ERK signaling in response to dehydration.

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REFERENCES

Aggeli, I. S., Gaitanaki, C., Lazou, A. and Beis, I. (2001). Activation of multiple MAPK pathways (ERKs, JNKs, p38-MAPK) by diverse stimuli in amphibian heart. *Mol. Cell. Biochem.* **221**, 63-69.

Anjum, R. and Blenis, J. (2008). The RSK family of kinases: emerging role in cellular signaling. *Mol. Cell. Biol.* **9**, 747-758.

Balinsky, J. B., Mavis, M. C. and Baldwin, E. (1961). The adaptation of amphibian waste nitrogen excretion to dehydration. *Comp. Biochem. Physiol.* **3**, 236-244.

Balinsky, J. B., Choritz, E. L., Coe, C. G. L. and Van Der Schans, G. S. (1967). Amino acid metabolism and urea synthesis in naturally aestivating *Xenopus laevis*. *Comp. Biochem. Physiol.* **22**, 59-68.

Beck, C. W. and Slack, J. M. (2001). An amphibian with ambition: a new role for *Xenopus* in the 21st century. *Genome Biol.* **2**, REVIEWS1029.

Benekli, M., Baer, M. R., Baumann, H. and Wetzler, M. (2003). Signal transducer and activator of transcription protein in leukemias. *Blood* **101**, 2940-2954.

Choi, H. J., Chung, Y. S., Kim, H. J., Moon, U. Y., Choi, Y. H., Van Seuningen, I., Baek, S. J., Yoon, H. G. and Yoon, J. H. (2009). Signal pathway of 17beta-estradiol-induced MUC5B expression in human airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **40**, 168-178.

Chung, J., Uchida, E., Grammer, T. C. and Blenis, J. (1997). Stat3 serine phosphorylation by ERK-dependent and independent pathways negatively modulates its tyrosine phosphorylation. *Mol. Cell. Biol.* **17**, 6508-6516.

Churchill, T. A. and Storey, K. B. (1993). Dehydration tolerance in wood frogs: a new perspective on the development of amphibian freeze tolerance. *Am. J. Physiol.* **265**, 1324-1332.

Cowan, K. J. and Storey, K. B. (2003). Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. *J. Exp. Biol.* **206**, 1107-1115.

English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu. S. and Cobb, M. H. (1999). New insights into the control of MAP kinase pathway. *Exp. Cell Res.* **253**, 255-270.

Gaitanaki, C., Konstantina, S., Chrysa, S. and Beis, I. (2003). Oxidative stress stimulates multiple MAPK signalling pathways and phosphorylation of the small HSP27 in the perfused amphibian heart. *J. Exp. Biol.* **206**, 2759-2769.

Greenway, S. C. and Storey, K. B. (2000a). Activation of mitogen-activated protein kinases during natural freezing and thawing in the wood frog. *Mol. Cell. Biochem.* **209**, 29-37.

Greenway, S. C. and Storey, K. B. (2000b). Mitogen-activated protein kinases and anoxia tolerance in turtles. *J. Exp. Zool.* **287**, 477-484.

Hillman, S. S., Withers, P. K., Drewes, R. C. and Hillyard, S. D. (2009). *Ecological and Environmental Physiology of Amphibians*. Oxford: Oxford University Press.

Holden, C. P. and Storey, K. B. (1997). Second messenger and cAMP-dependent protein kinase responses to dehydration and anoxia stresses in frogs. *J. Comp. Physiol. B* **167**, 305-312.

Iliodromitis, E. K., Gaitanaki, C., Lazou, A., Aggeli, I., Gizas, V., Bofilis, E., Zoga, A., Beis, I. and Kremastinos, D. T. (2006). Differential activation of mitogen-activated protein kinases in ischemic and nitroglycerin-induced preconditioning. *Basic Res. Cardiol.* **101**, 327-335.

Janssens, P. A. (1964). Urea production and transaminase activity in *Xenopus laevis* (Daudin). *Comp. Biochem. Physiol.* **13**, 217-224.

Krishna, M. and Narang, H. (2008). The complexity of mitogen-activated protein kinases (MAPKs) made simple. *Cell Mol. Life Sci.* **65**, 3525-3544.

Kultz, D. and Burg, M. (1998). Evolution of osmotic stress signaling *via* MAP kinase cascades. *J. Exp. Biol.* **201**, 3015-3021.

Larade, K. and Storey, K. B. (2006). Analysis of signal transduction pathways during anoxia exposure in a marine snail: a role for p38 MAP Kinase and downstream signaling cascades. *Comp. Biochem. Physiol. B* **143**, 85-91.

MacDonald, J. A. and Storey, K. B. (2004). Tyrosine phosphorylation and the control of cellular information. In *Functional Metabolism: Regulation and Adaptation* (ed. K. B. Storey), pp. 125-151. Hoboken, NJ: Wiley-Liss.

MacDonald, J. A. and Storey, K. B. (2005). Mitogen-activated protein kinase and selected downstream targets display organ-specific responses in the hibernating ground squirrel. *Int. J. Biochem. Cell Biol.* **37**, 679-691.

Matsuda, S., Kawasaki, H., Moriguchi, T., Gotoh, Y. and Nishida, E. (1995). Activation of protein kinase cascades by osmotic shock. *J. Biol. Chem.* **270**, 12781-12786.

May, L. T. and Hill, S. J. (2008). ERK phosphorylation: spatial and temporal regulation by G protein-coupled receptors. *Int. J. Biochem. Cell Biol.* **40**, 2013-2017.

Ning, Z. Q., Li, J. and Arceci, R. J. (2001). Signal transducer and activator of transcription 3 activation is required for Asp816 mutant c-Kit-mediated cytokine-independent survival and proliferation in human leukemia cells. *Blood* **97**, 3559-3567.

Price, N. M. and Harrison, P. J. (1987). Comparison of methods for the analysis of dissolved urea in seawater. *Mar. Biol.* **94**, 907-317.

Ramos, J. W. (2008). The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. *Int. J. Biochem. Cell Biol.* **40**, 2707-2719.

Richards, S. A., Dreisbach, V. C., Murphy, L. O. and Blenis, J. (2001). Characterization of regulatory events associated with membrane targeting of p90 ribosomal S6 kinase 1. *Mol. Cell. Biol.* **21**, 7470-7480.

Roux, P. P. and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* **68**, 320-344.

Ruvinsky, I. and Meyuhas, O. (2006). Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem. Sci.* **31**, 342-348.

Schaeffer, H. J. and Weber, M. J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell Biol.* **19**, 2435-2444.

Schwab, M. S., Kim, S. H., Terada, N., Edfjall, C., Kozma, S. C., Thomas, G. and Maller, J. L. (1999). P70s6k Controls selective mRNA translation during oocyte maturation and early embryogenesis in *Xenopus laevis*. *Mol. Cell. Biol.* **19**, 2485-2494.

Seiter, P., Schultheiss, H. and Hanke, W. (1978). Osmotic stress and excretion of ammonia and urea in *Xenopus laevis*. *Comp. Biochem. Physiol. A* **61**, 571-576.

Sridhar, S. S., Hedley, D. and Sit, L. L. (2005). Raf kinase as a target for anticancer therapeutics. *Mol. Cancer. Ther.* **4**, 677-685.

Tinsley, R. C. and Kobel, H. R. (1996). *The Biology of Xenopus*. Oxford: Oxford University Press.

Wu, X., Noh, S. J., Zhou, G., Dixon, J. E. and Guan, K. (1996). Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated Hela cells. *J. Biol. Chem.* **271**, 3265-3271.