Activation of mitogen-activated protein kinase by heat shock treatment in *Drosophila*

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Heat shock treatment of *Drosophila melanogaster* tissue culture cells causes increased tyrosine phosphorylation of several 44 kDa proteins, which are identified as *Drosophila* mitogen-activated protein (MAP) kinases. Tyrosine phosphorylation occurs within 5 min, and is maintained at high levels during heat shock. It decreases to basal levels during recovery, concurrent with the repression of heat shock transcription and heat-shock-protein synthesis. The increased MAP kinase tyrosine phosphorylation is paralleled by increased MAP kinase activity.

At least two MAP kinases, DmERK-A and DmERK-B, are identified whose tyrosine phosphorylation increases during heat shock. Thus MAP kinase activation is an immediate early response to heat shock, and its increased activity is maintained throughout heat shock treatment. Protracted MAP kinase activation may contribute to heat shock transcription factor phosphorylation and the numerous metabolic alterations that constitute the heat-shock response.

INTRODUCTION

Heat shock causes a dramatic reprogramming of metabolic activity. Many normal pathways are drastically repressed, including normal transcription and translation (reviewed by Lindquist [1]). Concurrently, a small subset of genes, the heat-shock genes, are very efficiently transcribed, processed and translated [1]. The first characterized event initiating the heat-shock-response pathway is the activation of the heat-shock transcription factor (HSF) [2]. In this report we provide evidence that heat shock treatment of *Drosophila* cells activates mitogen-activated protein (MAP) kinases, a ubiquitous family of cell signalling protein kinases implicated in numerous extracellular response systems. The activation characteristics are relatively unique, involve multiple forms of MAP kinase and closely parallel the activated state of HSF.

MAP kinases [also called extracellular-signal-regulated kinases (ERKs)] serve as a primary link between extracellular ligand-stimulated responses and altered transcription and translation factor activities [3–5]. MAP kinase variants have molecular masses of between 42 and 45 kDa, and become rapidly phosphorylated on tyrosine in response to a variety of extracellular activators (see Cobb et al. [6] for a comprehensive listing). Most are mitogens, such as fibroblast growth factor, epidermal growth factor (EGF), platelet-derived growth factor, phorbol 12-myristate 13-acetate (PMA) and insulin, but non-mitogenic activators have also been observed, for example, microtubule depolymerizers (colchicine, vinblastine) and KCl. MAP kinases are critical mediators of cell signalling throughout the eukaryotic kingdom, from yeast [7] to sea stars [8] to mammalian cells. A major molecular chain linking cell surface receptor activation to MAP kinase via Ras and subsequent

activation of an upstream kinase cascade [including Raf and Map kinase kinase (MEK)] has been recently delineated [9–12]. Activated MAP kinase phosphorylates cytoplasmic (pp90^{ras} [13–15]) and nuclear (*c-jun* [16,17]) regulators of gene expression (other substrates have also been identified). MAP kinase activated by other pathways, some independent of Ras activation, has also been observed [18,19].

The activation of MAP kinase by heat shock suggests that this event regulates aspects of the intensively investigated heat-shock response. A detailed analysis of the kinetics of MAP kinase activation and deactivation reveal a close temporal correlation with the activation and deactivation of HSF, supporting the view that MAP kinase activation contributes to HSF activation, and may be an early event involved in initiating the heat-shock response. During the period that these investigations were in progress, several reports have detailed the activation of MAP family kinases by heat shock [20,21]; MAP family kinases are also activated by interleukin 1 [22]. However, the several species that we identify in *Drosophila* present different molecular properties, and the elucidation of their role in *Drosophila* has special importance given the pre-eminent role that this organism plays in the molecular characterization of all aspects of the heat-shock response [1].

EXPERIMENTAL

Cell culture, heat shock and lysate preparation

Drosophila Schneider S2 cells were grown in 10% fetal calf serum at room temperature (22–24 °C) in T flasks (Corning). Experimental cells were transferred to suspension culture in scintillation vials containing stir fleas for agitation. Cells were heat-

Abbreviations used: HSF, heat-shock transcription factor; MAP kinase, mitogen-activated protein kinase; 1D, one-dimensional; 2D, two-dimensional; IEF, isoelectric focusing; PVDF, poly(vinylidene difluoride) membrane; ERK, extracellular-signal-regulated kinase; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; MEK, MAP kinase kinase; MBP, myelin basic protein.

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shocked by immersion in a circulator temperature-controlled water bath, with the temperature monitored by a digital thermometer to $\pm 0.1^\circ\text{C}$. Cells labelled with 2.5 mCi/ml [^{32}P]P_i were incubated in phosphate-lacking Dulbecco's modified Eagle's medium for 3.5 h. Half the culture was subsequently heat-shocked at 37°C for 30 min, while the other half was maintained at $22\text{--}24^\circ\text{C}$. Cell lysates were prepared before, during and after heat shock at 37°C using the following basic method: about 5×10^6 cells were removed from the suspension cultures, pipetted into 4°C wash buffer (50 mM KCl, 15 mM MgSO₄, 4 mM CaCl₂, 3 mM K₂HPO₄, 10 mM dextrose, 20 mM Hepes, pH 6.8) and washed twice by centrifugation in a clinical centrifuge. Cells were resuspended in 100 μl of hSDS (0.3% SDS, 1% 2-mercaptoethanol, 50 mM Tris/HCl, pH 8.0, kept at 100°C until added to cells). After cooling on ice for 1 min, 10 μl of DNase/RNase (1 mg/ml DNase, 0.5 mg/ml RNase, 50 mM MgCl₂ in 0.5 M Tris/HCl, pH 7.0) was added. Viscosity was dispersed by pipetting. Cell lysates for one-dimensional (1D) SDS/PAGE were mixed with 4 $\times$ Laemmli sample buffer; lysates for two-dimensional (2D) isoelectric focusing (IEF)/SDS/PAGE were mixed with urea (1 mg/ μl of lysate), and dissolved by vortexing. 1D gels were standard Laemmli formation, run at 20 mA gel; 2D gel electrophoresis was performed as described [23]. Another method of lysate preparation used routinely in the laboratory was also tested. Washed cells were lysed in 100 μl of TXC/LSB (25 mM Hepes, pH 7.2, 65 mM potassium acetate, 0.5 mM magnesium acetate, containing 0.5% Triton X-100). After microcentrifugation for 1 min, supernatant was collected. Substantially less immunoreactive 44 kDa protein was detected using the TXC/LSB lysis procedure.

Immunoblotting

After SDS/PAGE (10% gels), proteins were electrophoretically transferred to poly(vinylidene difluoride) (PVDF) matrix (Immobilon P; Millipore) in buffer containing 25 mM Tris base, 192 mM glycine, 10% methanol and 0.01% SDS for 5 h at 300 mA. Antigen was detected using the Amersham ECL Western Blotting Detection kit, according to the manufacturer's instructions. Incubation with primary antibody was for 2 h, followed by 1 h with secondary antibody [1:30000 dilution of anti-mouse horseradish peroxidase]. 4G10 antibody, a mouse monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology Inc.), was used at a dilution of 1:1000. Anti-DmERK-A and -B sera were used at 1:5000 dilution. Blots were blocked with either 5% milk (Carnation) or 5% BSA for probing with 4G10 or DmERK antisera respectively. The extent of MAP kinase activation (phosphorylation) varied severalfold between different cell lines. The specificity of the 4G10 antibody for phosphotyrosine was verified by demonstrating that 5 mM phosphotyrosine blocked immunoreactivity. Similar analyses of 4G10 specificity in *Drosophila* cells have been carried out by Biggs and Zipursky [24]. For reprobing with different primary antibodies, blots were stripped by immersion in 62.5 mM Tris/HCl, pH 6.7, containing 100 mM 2-mercaptoethanol and 2% SDS at 50°C for 30 min. The co-localization of protein(s) detected by the 4G10 antibodies and the DmERK antisera was determined by overlay of the two films. Identification of ^{32}P -labelled proteins as antigenically detected proteins was accomplished by electrotransferring the ^{32}P -labelled proteins resolved by 2D IEF/SDS/PAGE to PVDF, obtaining an autoradiogram of the immobilized proteins, then cutting out the appropriate sector of the gel and immunodetecting DmERK isoforms. The ECL- and ^{32}P -exposed films were superimposed for identification.

Affinity-chromatographic purification of phosphotyrosine-containing proteins

Preparation and use of 4G10-coupled affinity resin was as described by Torres et al. [25]. Briefly, cell lysates, prepared in hSDS, were diluted with 2 vol. of $1.5 \times$ column buffer (50 mM Tris/HCl, pH 7.5, 225 mM NaCl, 3 mM EDTA, 3 mM EDTA, 37.5 mM β -glycerophosphate, 1.5 mM sodium orthovanadate, 15 $\mu\text{g}/\text{ml}$ leupeptin, 15 $\mu\text{g}/\text{ml}$ aprotinin, 37.5 mM NaF). Lysates were loaded on to columns, incubated for 2 h at 4°C , washed with 2×10 ml of column buffer, and the bound proteins eluted with 100 nM *p*-nitrophenyl phosphate.

Determination of kinase activity

Peptide-phosphorylation assay

Phosphorylation of the T-669 peptide (ELVEPLTPSGEAPNQ-ALLRKK, corresponding to the Thr-669 phosphorylation site on the EGF receptor) was determined as described by Torres et al. [25]. About 5×10^7 S2 cells (control and those treated for 30 min at 37°C) were washed twice in wash buffer and homogenized in 1 ml of lysis buffer (100 mM Tris/acetate, pH 7.5, 100 mM magnesium acetate, 0.1% Triton X-100, 25 mM NaF, 0.2 mM sodium orthovanadate, 100 $\mu\text{g}/\text{ml}$ soya-bean trypsin inhibitor, 20 mM benzamidine, 5% glycerol). After microcentrifugation for 1 min, the supernatants were collected and kinase activity was measured immediately. Each activity determination used 20 μl of cell lysate, 400 μM T-669 in 100 mM Tris/acetate buffer, pH 7.5, in a final volume of 50 μl . Reaction was initiated by adding 5 μl of 1 mM [γ - ^{32}P]ATP in 100 mM magnesium acetate. The specific radioactivity of [γ - ^{32}P]ATP was 100 c.p.m./pmol. After incubation at 30°C for 10 min, the reaction was stopped by adding 5 μl of phosphoric acid. The reaction mixture was microcentrifuged for 5 min, and the supernatant was transferred to P-81 phosphocellulose paper. The paper was washed 2×5 min in 1% phosphoric acid, 2×5 min in water, air-dried and counted. Non-specific binding/kinase activity (background) was measured by incubation without T-669 for 10 min at 30°C , and subtracted from experimental values. Background incorporation and normal-temperature cell incorporation were similar, resulting in a very low specific incorporation. Consequently, the fold increase cannot be accurately quantified, but clearly is greater than 5–10-fold. Kinetic analysis revealed a time-dependent increase in specific T-669 phosphorylation. Phosphorylation was also measured after precipitation of the reaction mixture. Peptide was virtually all recovered in the supernatant. The quantification produced very similar numbers to the spotted whole reaction mixture.

In vitro myelin basic protein (MBP)-phosphorylating activity with and without immunoprecipitation

Cells were washed, then lysed in IPB (40 mM sodium pyrophosphate, 50 mM NaF, 5 mM MgCl₂, 20 mM Hepes, pH 8.0, 10 μM sodium orthovanadate, 10 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 $\mu\text{g}/\text{ml}$ leupeptin, 10 mM benzamidine, 100 $\mu\text{g}/\text{ml}$ soya-bean trypsin inhibitor), and the cytoplasmic portion recovered after microcentrifugation for 5 min at 4°C . About 50 μg of protein was incubated with DmERK antiserum at 4°C for 2 h, with intermittent mixing. Protein A-Sepharose (Pharmacia) (15 μl) was added, and incubation continued for 1 h with mixing. The immune complexes were recovered by microcentrifugation for 1 min, washed twice with RIPA buffer (10 mM Hepes pH 7.2, 70 mM NaCl, 0.1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.05% 2-mercaptoethanol), twice with kinase assay buffer (KAB: 30 mM

Hepes, pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP), then resuspended in 30 μl of KAB containing 0.4 μCi of $[\gamma\text{-}^{32}P]ATP$ and 15 μg of MBP and incubated for 30 min at 30 °C. Then 15 μl of 4 $\times$ SDS sample buffer was added, the mixture was heated at approx. 98 °C for 4 min, the Protein A–Sepharose was removed by microcentrifugation for 1 min, and the supernatant applied to an SDS/14% polyacrylamide gel. For analysis of MBP phosphorylation without immunoprecipitation, lysates were separated in KAB by three successive freeze–thaw cycles, and approx. 50 μg of protein was incubated with 0.4 μCi of $[\gamma\text{-}^{32}P]ATP$ and 15 μg of MBP as described above.

In situ analysis of MBP-phosphorylating activity

Cells were washed, then lysed in TXC/LSB with protease inhibitors as described above by two successive freeze–thaw cycles; 1 mg of urea was added per μl of lysate volume. SDS/polyacrylamide gels were polymerized with 130 μg of MBP/10 ml. After electrophoresis, gels were fixed in 50 mM Tris/HCl (pH 8.0)/20% propan-2-ol for 2 $\times$ 30 min, and then incubated for 60 min in 50 mM Tris/HCl (pH 8.0)/5 mM 2-mercaptoethanol. They were further incubated in 6 M guanidinium chloride for 60–120 min, then washed with five changes of 250 ml of 50 mM Tris/HCl pH 8.0, containing 5 mM 2-mercaptoethanol and 0.04% Tween 20 over 15 h at 4 °C. Another incubation followed in reaction buffer (40 mM Hepes, pH 8.0, 2 mM 2-mercaptoethanol, 0.1 mM EGTA, 5 mM $MgCl_2$, 2 mM $MnCl_2$) for 30 min, then 30 min in a fresh 10 ml solution of reaction buffer containing 10 μM ATP and 100 μCi of $[\gamma\text{-}^{32}P]ATP$. Finally, gels were washed five times with 500 ml of 5% trichloroacetic acid/1% sodium pyrophosphate over 5 h, stained with Coomassie Brilliant Blue, dried under vacuum on Whatman 3 MM, and exposed to autoradiographic film for 4–15 days.

RESULTS

Heat-induced tyrosine phosphorylation of a 44 kDa protein

To identify potential signal-transduction molecules activated by heat shock, proteins showing changes in tyrosine phosphorylation were identified. Lysates from *Drosophila* tissue culture cells were prepared before and after a 37 °C 30 min heat shock, and characterized by immunoblot analysis using anti-phosphotyrosine antibody (4G10). Several reproducible changes in phosphotyrosine immunoreactivity were detected. The most prominent change was the dramatically enhanced reactivity of a 44 kDa protein (Figure 1a). This enhanced reactivity was optimized when proteins were extracted by a 100 °C SDS procedure (results not shown; see the Experimental sections for discussion). Appropriate controls were carried out to verify that the 4G10 antibodies were specific for phosphotyrosine (see the Experimental section). Several additional but less pronounced heat-shock-induced changes in phosphotyrosine content were also reproducibly detected. An example is indicated in Figure 1(b). Other examples include a marked loss of phosphotyrosine on 68 and 85 kDa proteins during recovery (Figure 1b, lanes 6 and 7). Other tyrosine-phosphorylation changes observable in Figure 1 (e.g. enhanced phosphorylation of an approx. 53 kDa band) were not reproducibly detected.

Extracts were next analysed by 2D IEF/SDS/PAGE and 4G10 immunoblotting (Figure 2). The single heat-enhanced p44 band was resolved into two widely separated prominently induced spots ($pI_1 \sim 5.7$, $pI_2 \sim 6.2$). The pI 5.7 and 6.2 spots are labelled ERK-A and ERK-B respectively on the basis of further characterization described in succeeding sections. A third minor more basic heat-shock-enhanced tyrosine-phosphorylated 44 kDa

Figure 1 Tyrosine phosphorylation of a 44 kDa protein after heat shock

Protein lysates were prepared from control and heat-shocked (30 min, 37 °C). *Drosophila* S2 tissue culture cells and cells heat-shocked and allowed to recover at normal temperature. Equivalent amounts of protein (corresponding to equivalent cell numbers) were loaded in each lane (verified by Amido Schwartz staining before immunodetection). Phosphotyrosine-containing proteins were identified by immunoblotting and probing with 4G10 anti-phosphotyrosine antibodies. (a) Control (lane 1) and heat-shocked (lane 2) cells. A protein with substantially diminished tyrosine phosphorylation is indicated by the open arrow. The location of the principal induced tyrosine phosphorylation, a 44 kDa protein, is indicated by a solid arrowhead (and in b). (b) Lane 1, control; lane 2, heat-shocked for 2 min; lane 3, heat-shocked for 5 min; lane 4, heat-shocked for 15 min; lane 5, heat-shocked for 30 min; lane 6, heat-shocked for 30 min and allowed to recover for 1 h; lane 7, as lane 6 but 2 h recovery time.

protein was also detected. Its tyrosine phosphorylation increased in most, but not all, heat-shocked samples. The disparate pI s strongly suggest that these are different proteins of approximately equivalent molecular mass, not differentially phosphorylated versions of the same protein. Results described below confirm this interpretation. In some analyses, such as the one shown in Figure 2, ERK-A was separated into two closely spaced variants, suggesting that two tyrosine-phosphorylated versions occur.

The 44 kDa proteins are *Drosophila* MAP kinase isoforms

The molecular mass and rapid activation of these prominent tyrosine-phosphorylated proteins suggested that one or more might be *Drosophila* MAP kinase(s). One *Drosophila* MAP kinase, termed DmERK-A, has recently been cloned by Biggs and Zipursky [24]. Using antibodies raised to a synthetic peptide (see ref. [24] for a description), immunoblot analysis of 1D and 2D gels was carried out. The 1D analysis detected a single major ERK-A band of ~ 44 kDa (Figure 3a). A minor band of 55 kDa was also detected. DmERK-A and the 44 kDa heat-enhanced 4G10-detected band precisely co-migrated on 1D PAGE, based on stripping and reprobing a single blot. The abundance of *Drosophila* ERK-A was not altered by heat shock. The 2D analysis resolved three to four spots of $pI \sim 5.7\text{--}5.8$ (labelled 1–4 in Figure 3d; the minor form 4 spot intensity varied, from undetectable to being equal to or greater than form 3). The pI s of the spots differ by small amounts, suggesting that they

Figure 2 The 44 kDa induced protein band is comprised of two distinct species

Protein lysates were prepared from control and heat-shocked (30 min, 37 °C) *Drosophila* S2 tissue culture cells as above, resolved by 2D IEF/SDS/PAGE, and phosphotyrosine-containing proteins identified by immunoblotting. Equivalent amounts of protein (corresponding to equivalent cell numbers) were loaded on each first-dimensional gel (verified by Amido Schwartz staining before immunodetection). (a) Control cells; (b) heat-shocked cells. The locations of the two major 44 kDa proteins (ERK-A and ERK-B) are indicated; the minor pI 6.6 heat-enhanced (and anti-DmERK-B-reactive) spot is indicated by an arrowhead (see the text for discussion).

represent DmERK-A containing zero, one, two and three phosphates per molecule. Forms 3 and 4 migrated to the same 2D co-ordinates as the pI 5.7 4G10-detected phosphotyrosine-containing proteins (as labelled in Figure 2, and further detailed below).

The abundance of the more phosphorylated DmERK-A spots (Forms 3 and 4) increased significantly after heat shock (compare Figures 3c and 3d, showing non-heated and heat-shocked lysates respectively). The fraction of ERK-A in tyrosine-phosphorylated forms increased from $10 \pm 4\%$ to $38 \pm 8\%$, based on the average of three independent determinations. Since the immunoblots quantify protein amount, these numbers directly reflect the stoichiometry of phosphorylation.

To show unambiguously that the pI ~ 5.7 heat-enhanced 4G10-detected phosphotyrosine-containing protein spots were ERK-A, two further experiments were performed. First, single blots were sequentially probed with both 4G10 and anti-DmERK-A antibodies. DmERK-A forms 3 and 4 precisely co-migrated with the pI 5.7 4G10-detected spots. Since the form 2 ERK-A spot did not react with the 4G10 anti-phosphotyrosine antibodies, we presume that MAP kinase con-

Figure 3 Detection of *Drosophila* ERK-A (MAP kinase A) by anti-DmERK-A serum

(a) Equal amounts of protein from control (lane 1), heat-shocked (lane 2) and recovered (lane 3) cells were analysed by 1D SDS/PAGE and immunoblotting using anti-DmERK-A serum [13]. The predominant 44 kDa band (ERK-A; arrow) as well as a minor ~ 55 kDa band are evident. (b) A heat-shocked lysate was resolved by 2D IEF/SDS/PAGE, and ERK-A identified by immunoblotting. (c) and (d). Parallel-prepared control (c) and heat-shocked (30 min, 37 °C) (d) lysates were analysed by 2D IEF/SDS/PAGE and immunoblotting as above. Equivalent amounts of protein (corresponding to equivalent cell numbers) were loaded on each first-dimension gel (verified by Amido Schwartz staining before immunodetection). The position of the four ERK-A isoforms are marked in (d) and form 2 is marked by a line in both (b) and (c). Only a portion of the 2D immunoblot is shown in (c) and (d). The dots are pen marks used to orient the ECL-detected DmERK-A to Amido Schwartz-stained abundant cell proteins on the blots.

taining only tyrosine phosphate does not occur; tyrosine phosphorylation occurs only subsequent to or concurrently with a 'priming', presumably threonine, phosphorylation. The detection of four spots suggests there may be an additional third phosphorylation site in the *Drosophila* protein. The abundance of form 4 varied greatly from analysis to analysis, ranging from less than 10% to 20–30% of the total immunoreactive protein.

The increased tyrosine phosphorylation of *Drosophila* MAP kinase during heat shock is consistent with the heat shock causing MAP kinase activation. Both threonine and tyrosine phosphorylation probably occur, on the basis of IEF results, which would parallel mammalian MAP kinase where both phosphorylations are required for activation [26–28]. The molecular masses of the putative phosphorylated forms did not increase (in our gel system), in contrast with studies of mammalian kinase where tyrosine-phosphorylated MAP kinase was about 2 kDa heavier.

Verification by sequential immunoaffinity chromatography and immunoblotting that MAP kinase is tyrosine-phosphorylated during heat shock

In a second experimental approach to verify that the pI 5.7 4G10-detected spot is DmERK-A, cell extracts were analysed in a two-step procedure. First, phosphotyrosine-containing proteins were immunopurified on a 4G10 affinity column. Second, the immunopurified proteins were analysed by 1D immunoblotting

Figure 4 Purification of MAP kinase from heat-shocked cells by affinity chromatography on an anti-phosphotyrosine antibody column

Lysates were prepared, passed over the affinity column, and proteins recovered by *p*-nitrophenyl phosphate elution. (a) Analysis of the purified proteins by immunoblotting with MAP kinase antiserum; (b) analysis of the purified proteins by immunoblotting with 4G10 anti-phosphotyrosine antiserum. Affinity-purified proteins were from control (lane 1) and heat-shocked (lane 2) lysates.

with DmERK-A antiserum. DmERK-A-reactive protein was detected in the immunopurified proteins from heat-shocked cells (Figure 4a), and at substantial higher levels as predicted if DmERK-A becomes more heavily tyrosine-phosphorylated during heat shock. Reprobing the immunopurified proteins with the 4G10 antibody detected the expected darker 44 kDa band in the heat-shocked sample (Figure 4b, lane 2). Thus all lines of evidence indicate that DmERK-A tyrosine phosphorylation increases during heat shock of *Drosophila* cells in tissue culture.

One or more of the other heat-induced 44 kDa tyrosine-phosphorylated proteins is DmERK-B

Several MAP kinase isoforms have been identified in mammalian and yeast cells [7,29], and Biggs and Zipursky [24] identified several related 'kinase' sequences in DNA fragments generated by PCR using primers to conserved segments of MAP-family kinases. To investigate whether the other 44 kDa 4G10-detected spots (major pI 6.2, minor pI 6.6) were other MAP kinase

Figure 5 Detection of *Drosophila* MAP kinases with anti-DmERK-B serum

Equal amounts of protein from control and heat-shocked cells were analysed by IEF/SDS/PAGE and immunoblotting, and detected using anti-DmERK-B serum. (a) Non-heat-shocked cells; (b) heat-shocked (30 min, 37 °C) lysates. The blots were subsequently probed with the 4G10 antibody. The location of the 4G10-reactive spots in heat-shocked cells, which precisely co-migrated with certain DmERK-B-reactive spots, is indicated by arrows in (b). Only a portion of the 2D immunoblot is shown. The pI 6.2 cluster, composed of one major and two minor spots, is to the left, in each panel; the pI 6.6 cluster, composed of two major and two minor spots, is to the right.

Figure 6 Identification of MAP kinases as phosphoproteins in ^{32}P -labelled lysates

Drosophila cells were labelled with ^{32}P as described in the Experimental section; lysates were prepared and resolved by IEF/SDS/PAGE, electrophoretically transferred to PVDF, followed by autoradiography (3 days at 24 °C) and immunodetection with DmERK-A and DmERK-B antibodies. Equivalent amounts of protein (corresponding to equivalent cell numbers) were loaded on each first D-dimensional gel (verified by Amido Schwartz staining before immunodetection). The autoradiograms from non-heat shocked (a) and heat-shocked (b) cells are shown, with the location of the DmERK-A co-migrating phosphoprotein indicated. Insets: the blots were subsequently probed with DmERK-A antibodies to localize DmERK-A isoforms. The heat-enhanced form is indicated. The dots are pen marks used to orient the blots to the autoradiogram. Slight differences in DmERK-A spot distribution (cf. Figure 3) have been observed in independent experiments. No ERK-B co-migrating ^{32}P -labelled spots could be detected.

isoforms, immunoblot analysis using peptide-specific antisera to a second ERK-family kinase, termed DmERK-B (W. H. Biggs and S. L. Zipursky, personal communication) was performed. This anti-peptide antibody was used to probe 2D gel-resolved *Drosophila* proteins from control and heat-shocked cells, and subsequently the blots were reprobed with anti-phosphotyrosine 4G10 antibodies to determine which, if any, spots co-localized. Anti-DmERK-B immunoblots (Figure 5) are significantly more complex than DmERK-A blots, and require careful explication. However, the final conclusion is that both the major (pI 6.2) and

Figure 7 MAP kinase activity is increased by heat shock

Protein lysates were prepared from cells before, during and after heat shock, and analysed for MAP kinase activity as described in the Experimental section. (a) Specific phosphorylation quantified using a peptide substrate (T-669) corresponding to the phosphorylation site of the EGF receptor. Equivalent amounts of protein (corresponding to equivalent cell numbers) were tested for kinase activity in all cases. C, Before heat shock; HS_n, length (min) of heat shock at 37 °C; R_n, length (min) of recovery at 22–24 °C after a 30 min 37 °C heat shock. Absolute values (³²P c.p.m. incorporated) varied substantially in individual experiments, but the more than 5-fold increase in activity was consistently observed in replicate analyses. (b) MBP-phosphorylating activity, determined using either whole cell lysates (lane 1, before heat shock; lane 2, 30 min at heat shock at 37 °C) or immunopurified proteins (lanes 3 and 5, before heat shock; lanes 4 and 6, 30 min heat shock at 37 °C). Lysate proteins were precipitated with either DmERK-A antibodies (lanes 3 and 4) or DmERK-B antibodies (lanes 5 and 6). Equivalent amounts of protein were used for the whole cell lysate assay and the immunoprecipitation step. (c), (d) MBP-phosphorylating activity, determined by *in situ* assay with MBP polymerized in the gel. Protein lysates were prepared from cells before (b) and after (d) 30 min heat shock at 37 °C, and analysed as described in the Experimental section. Equivalent amounts of protein (corresponding to equivalent cell numbers) were loaded on each first-dimension gel. (e) Coomassie Brilliant Blue-stained pattern of heat-shocked proteins (autoradiograph shown in d) with the location of the 44 kDa MBP-phosphorylating activity indicated by arrow.

minor (pI 6.6) 4G10 spots are detected by the anti-DmERK-B serum, indicating that one or both heat-enhanced phosphotyrosine proteins are DmERK-B, as discussed below.

Anti-DmERK-B reacts with two groups of proteins, at pI approx. 6.2 and 6.6, with multiple spots in each grouping (Figure 5). In the pI 6.2 constellation (the left group in each panel) there is a single major spot, which does not react with 4G10 and whose abundance is not markedly altered by heat shock. There are additional minor spots, to the acidic (left) side. The most acidic spot is present at low levels in non-heated cells, and its abundance increases after heat shock (indicated with downward-pointing arrow in Figure 5b). This specific spot co-migrated with the prominent 4G10-detected pI 6.2 spot. Although the heat-induced form represents a very minor fraction of the total (as determined with DmERK-B antiserum), it either represents a significant amount of tyrosine-phosphorylated protein, based on its strong reaction with 4G10, or its phosphotyrosine-containing epitope is configured to react unusually strongly with the 4G10 antibody. Please note that the relative amounts of anti-DmERK (A and B)

immunoreactivity (spot intensity) cannot be equated with their absolute intracellular abundance, or that of their phosphorylated variants, as the relative sensitivities of the several antibodies used in this study differ.

The pI 6.6 cluster is even more complex. We usually detect two major spots in this constellation, with similar but differentiable molecular masses. The lower-molecular-mass form has two minor slightly more acidic partners, which we interpret as being two phosphorylated variants. The molecular mass increases slightly with increased phosphorylation, as has been described for mammalian ERK isoforms. The more acidic form (upward-pointing arrow in Figure 5b) precisely co-migrated with the minor pI 6.6 4G10-detected phosphotyrosine-containing protein. The upper major spot was only detected in some lysates; when present, a second substantially more acidic form was also observed (open arrowhead in Figure 5b), probably representing a phosphorylated variant. Although the acidic shift is significantly greater than observed for the DmERK isoforms, it is consistent with phosphorylation-based isoelectric point shifts previously

observed. The basis for different proteins shifting apparent pI to differing extents after a single phosphorylation has not been resolved, but has been frequently observed, and may reflect conformational perturbations due to phosphorylation influencing apparent pI .

The detection of several proteins with the DmERK-B anti-serum probably results from extensive domain similarity between differing, but closely related, isoforms. The antigenic peptide is probably common to the several isoforms, leading to the cross-reactivity. More extensive DmERK sequencing and the creation of isoform-specific antigenic peptides will be required to resolve the precise identities of these (presumably) related ERK isoforms.

Further evidence that the tyrosine-phosphorylated 44 kDa proteins are phosphorylated MAP kinases.

To document further the phosphorylation of MAP kinase by heat shock, cells were labelled with [^{32}P]P_i before and during heat shock, and the resultant ^{32}P -labelled phosphoproteins displayed by IEF/SDS/PAGE. A novel phosphoprotein spot can be detected in the heat-shocked lysate (indicated by an arrow in Figure 6b), which precisely co-migrated with the heat-shock-enhanced DmERK-A isoform, as assessed by subsequent immunoprobings of the same gel-resolved proteins [compare the insets in Figures 6a (non-heat shock) and 6b (heat shock); see the Experimental section for details]. This provides further confirmation that ERK-A isoforms are due to phosphorylation. No ^{32}P -labelling of form 2 could be detected, even though it is prominently represented in this sample (see insets to Figure 6), suggesting that this phosphate turns over relatively slowly or is not a phosphorylated variant. In addition, the more acidic heat-induced ^{32}P -labelled phosphoprotein just to the left of the heat-enhanced DmERK-A isoform on the autoradiogram (Figure 6b) does not correspond to an immunologically detectable DmERK-A isoform; its identity awaits further characterization. Immunoprecipitation of ^{32}P -labelled lysates with anti-DmERK-A antibodies showed enhanced DmERK-A phosphorylation during heat shock, but the specific isoelectric variants responsible for the enhanced labelling could not be determined because the immunoprecipitated proteins repeatedly failed to focus precisely on IEF/SDS/PAGE analysis. No detectable ^{32}P -labelled proteins migrated at the co-ordinates of any of the ERK-B isoforms, and immunoprecipitation with anti-DmERK-B did not detect ^{32}P -labelled protein; the abundance of phosphorylated DmERK-B is presumably too low to detect.

Tyrosine phosphorylation of MAP kinase during heat shock increases its activity

To demonstrate that tyrosine phosphorylation of MAP kinase resulted in increased activity, three complementary analyses were performed, each of which indicated that MAP kinase activity was enhanced by heat shock. First, *in vitro* phosphorylation assays were performed using a synthetic peptide substrate, T-669, which corresponds to the site of phosphorylation of mammalian EGF receptor (see the Experimental section for peptide sequence). Extracts were prepared from normal temperature and heat-shocked cells and the extent of T-669 phosphorylation was quantified using [γ - ^{32}P]ATP. MAP kinase phosphotransferase activity increased 5–10-fold during heat shock (Figure 7a). MAP kinase activity in non-heat-shocked cells was low, similar to background, rendering it difficult to quantify precisely the extent of heat-shock-induced activation (Table 1).

The kinetics of MAP kinase activation and tyrosine phosphorylation were also determined, to assess their correlation with the induction/inactivation of the heat-shock response.

Table 1 MAP kinase activity in control, heat-shocked and recovered samples

Protein lysates were prepared and analysed for MAP kinase-specific phosphorylation using a peptide (T-669) phosphate acceptor corresponding to the phosphorylation site of the EGF receptor, as described in the Experimental section. Equivalent amounts of protein (corresponding to equivalent cell numbers) were tested for kinase activity in all cases.

Incubation time/conditions	MAP kinase activity (pmol/10 min per 10 ⁶ cells)
Experiment 1	
Pre-heat shock	0.3
30 min at 37 °C	16.8
Experiment 2	
Pre-heat shock	0.1
5 min at 37 °C	7.8
30 min at 37 °C	16.3
30 min at 37 °C, 60 min at 23 °C	10.3
30 min at 37 °C, 120 min at 23 °C	3.3
30 min at 37 °C, 180 min at 23 °C	0.4

Activated HSF and heat-shock-protein mRNA and protein synthesis can be detected within 5–10 min of heating ([30]; F. Chen, M. Torres and R. F. Duncan, unpublished work). When heat-shocked cells are returned to their normal growth temperature of 22–24 °C, heat-shock transcription begins to decline after 1–2 h because of HSF inactivation ([31]; F. Chen, M. Torres and R. F. Duncan, unpublished work). MAP kinase phosphotransferase activity increases within 5–10 min, and remains high during the 30 min of heat shock treatment (Figure 7a). This represents a protracted interval of activation, compared with the transitory activations that characteristically follow extracellular receptor activation. However, protracted receptor-mediated activation has been observed in certain instances [32,33], and suggested to be required for nuclear transcription factor activation. During recovery from heat shock there is a slow decay of activity, with a half-life of about 1 h (Figure 7a). The changes in MAP kinase activity parallel the changes in tyrosine phosphorylation (Figure 1b). The 5 min interval before the initial analysis corresponds roughly to the length of time required to reach 37 °C under our heating conditions. The kinetics suggest that MAP kinase activation is an early primary signal in the heat-shock response. The parallel kinetics between the activation/inactivation of HSF [30,31] and MAP kinase are consistent with MAP kinase activation influencing the activity of HSF.

The second method of demonstrating MAP kinase activation by heat shock involved *in vitro* phosphorylation assays using MBP as the substrate. Using the whole cell lysates, a dramatic increase in MBP-phosphorylating activity could be detected [Figure 7b, compare lane 1 (before heat shock) with lane 2 (during heat shock)]. To show that the enhanced activity was specifically due to ERK activation, lysate proteins were immunoprecipitated with anti-DmERK-A and anti-DmERK-B antibodies and the immunoprecipitated pellet proteins were incubated with MBP and [γ - ^{32}P]ATP. The DmERK-A immunoprecipitate showed significantly enhanced MBP-phosphorylating activity after heat shock, whereas no detectable activation of DmERK-B was observed (Figure 7b, lanes 3–6). Quantitatively, the MBP band intensity was increased by 4.2-fold after heat shock, based on scanning densitometry (average of four determinations). By way of comparison, DmERK-A activity was increased by 7.3-fold at 5 min and 1.5-fold at 30 min in non-heat-shocked cells treated with insulin (average of three deter-

minations). PMA also increased MAP kinase activity by 1.5-fold (30 min treatment).

Finally, MBP-phosphorylating activity was measured using an *in situ* kinase assay with the MBP substrate polymerized into the gel. Lysates, before and after heat shock, were analysed by 2D electrophoresis so that the various pI MAP kinase variants would be individually resolved. A single 44 kDa heat-enhanced spot was detected [compare Figures 7c (before heat shock) and 7d (during heat shock)], which migrated at the co-ordinates characteristic of the DMERK-A form as assessed by Coomassie Brilliant Blue staining of the MBP-impregnated gel before drying and autoradiography (Figure 7e). A major uncharacterized approx. 150–200 kDa heat-enhanced MBP kinase was also observed, at a pI of about 6.4–6.6 (Figure 7d).

Thus, by all three criteria, DmERK-A is a heat-activated MAP kinase. Its kinetics are consistent with an early role in the heat-shock response, perhaps in the activation of the HSF. This intriguing possibility warrants further precise investigation.

DISCUSSION

MAP kinase activation has been identified as an immediate early necessary response to initiate intracellular responses to a variety of extracellular stimuli. It is best characterized as a prelude to mitogenesis, whence it derives its name, but has also been implicated in mating response, glycerol response and cell wall biosynthesis in yeast [7], in fertilization in invertebrates [8], in neuronal responses [33,34], and recently in the initiation of stress responses [20–22,35,36]. HSF activation is the first well-characterized change that occurs in the heat-stressed cell. Activation of HSF is temporally correlated with its phosphorylation [2,37,38], although the significance of this remains clouded. Activation also requires conversion from monomer into trimer [2,39,40]. It is unclear whether trimerization precedes phosphorylation, or vice versa, or whether the two events are functionally linked. The activation and repression of MAP kinase(s) during heat shock and subsequent recovery, concurrent with corresponding changes in HSF activity, are consistent with MAP kinase regulation of HSF activity via phosphorylation. This represents an intriguing and readily testable hypothesis. Consistent with this conjecture, the consensus site for MAP kinase phosphorylation, Pro-Xaa-Ser/Thr-Pro [41], is present in the middle portion of the HSF sequence. However, it is equally possible that MAP kinase activation targets other substrates with other consequences on the heat-shock response. These could include, for example, protein-synthesis-initiation factors influencing the heat-shock-translational response [42] or mRNA-degradatory factors that modulate mRNA stability [43].

There are several features of MAP kinase activation by heat shock in *Drosophila* that distinguish it from other systems and may be related to its specific role in the heat-shock response. Activation is protracted rather than transient. Similarly, in certain other systems, prolonged MAP kinase activation has been observed, and suggested to be a necessary condition for its translocation to nucleus and phosphorylation of target nuclear transcription factors [32,33]. Therefore the prolonged activation observed during heat shock may indicate a nuclear site of action and in particular reflect targeting of HSF. Heat shock causes multiple ERK isoforms to become tyrosine-phosphorylated, although in these studies only the DmERK-A isoform could be shown to have its activity increased. Activation in terms of tyrosine phosphorylation is substantial, easily detectable in unfractionated cell lysates as a major stimulated band. In contrast, insulin-dependent tyrosine phosphorylation of MAP kinase in *Drosophila* is transient and can only be detected by

immunopurification after a 30 min treatment ([24]; F. Chen, M. Torres and R. F. Duncan, unpublished work), although initial insulin-dependent MAP kinase activation was slightly greater than that caused by heat shock.

The heat-shock signal-transduction pathway by which HSF becomes activated leading to the pleiotropic battery of molecular changes that constitute the heat-shock response has remained enigmatic. Heat-induced alterations in membrane fluidity have been reported, which may lead to the activation of membrane-localized signalling elements [44,45] such as phospholipases, with concomitant formation of diacylglycerol and IP₃. Consistent with this conjecture, increased cytoplasmic IP₃ levels have been recorded [46], and PMA treatment has caused a modest heat-shock response in some experiments [47,48]. However, our attempts to detect PMA induction of heat-shock-gene transcription in *Drosophila* tissue culture cells have failed on virtually every occasion. Results reported here present an alternative possibility, implicating MAP-family kinase activation in the induction of the heat-shock response.

Aberrant proteins can induce the transcription of heat-shock genes [49,50], thus establishing a direct link between HSF activation and a diffusible intracellular molecule. This raises the possibility of MAP kinase activation via an intracellular sensor. However, heat-shock activation of HSF and MAP kinase may be distinct processes, elicited by distinct signal-transduction pathways, as MAP kinase activation is usually linked to membrane-localized sensor proteins, such as growth factor receptors, Ras, and Raf. However, the recent observation that MAP-family kinases are activated by protein-synthesis inhibitors such as anisomycin [20] would be consistent with a model whereby aberrant intracellular proteins constitute an activation signal.

Recently several groups have reported activation of MAP-family kinases by heat shock in mammalian cell types [20–22, 35, 36]. Thus MAP-family kinase activation appears to be a conserved response across the phylogenetic spectrum. However, one of the mammalian heat-shock-activated MAP kinases, termed SAPK or JNK-55 [51], has an atypically high molecular mass (~ 54 kDa [20]), and appears to be distinct from the 42–44 kDa forms, such as those we identify in *Drosophila*. The smaller mammalian heat-shock-activated MAP-family kinase variant, which is related to the *Saccharomyces cerevisiae* HOG1 MAP kinase variant and termed p38 or RK [21,22], has a molecular mass of ~ 38–42 kDa [21] and is activated by a unique upstream kinase (MKK3 [52]) distinct from ERK kinase (MEK1, MEK2 [21,22,52]). The amino acid separating the tripeptide dual-phosphorylation motif in MAP-family kinases provides a clue as to their type; DmERK-A has Thr-Gly-Tyr as does p38/RK, whereas ERK-type kinases typically have Thr-Glu-Tyr, suggesting that DmERK-A is a stress-activated-type MAP-family kinase. By analogy, activation of an upstream MKK3 homologue may occur during heat shock. However, DmERK-A was identified by homology with the ERK-type MAP kinases [24], is activated by well-characterized ERK activators such as insulin [24], and very efficiently phosphorylates the ERK substrate peptide T-669. These contrasting features suggest that DmERK-A may be activated by diverse stimuli and participate in ligand-activated as well as stress-activated signalling pathways.

There are additional heat-activated kinases which are downstream substrates of MAP kinase isoforms (based on studies in mammalian cells), and which may participate in the heat-shock response. For example, MAPKAP kinase 2 (MAP kinase-activated protein kinase 2) activity significantly increases after heat shock [53] leading to the phosphorylation of HSP27, the small mammalian heat-shock protein. Another likely downstream substrate is RNA polymerase II (C-terminal domain),

which is a good MAP kinase substrate [54,55] and is phosphorylated during heat shock, with potential consequences on transcriptional aspects of the heat-shock response. HSF may be yet another downstream MAP kinase target.

Finally, even if HSF phosphorylation increases after heat shock due to MAP kinase activation, this specific phosphorylation is unlikely to be a primary signal in the activation of stress-gene transcription. Many external stimuli that activate MAP kinase do not turn on HSF, although the magnitude of induction by heat seems far greater (discussed above) which may influence its effects on substrates such as HSF. HSF activation may be modulated by inputs from several signal transducers, with MAP kinase being one contributing component. To address this, current aims are to quantify the effect of ablating MAP kinase activity on the heat-shock response, and to determine directly whether MAP kinase phosphorylates HSF *in vitro*. Current attempts to address the first aim, via overexpression of a dominant negative version of MAP kinase, have thus far been thwarted by a lack of a uniformly expressing cell population and inadequate overexpression. However, experiments using drugs that activate or suppress MAP kinase (genistein, herbimycin, okadaic acid) show a positive correlation between activation or suppression of MAP kinase activity and enhancement or suppression of heat-shock-gene transcription respectively (F. Chen and R. F. Duncan, unpublished work).

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