The Mechanism of Heat Shock Activation of ERK Mitogen-activated Protein Kinases in the Interleukin 3-dependent ProB Cell Line BaF3*

Dominic C. H. Ng‡ and Marie A. Bogoyevitch‡§‡

We have investigated heat shock stimulation of MAPK cascades in an interleukin 3-dependent cell line, BaF3. Following exposure to 42°C, the stress-activated JNK MAPKs were phosphorylated and activated, but p38 MAPKs remained unaffected. Surprisingly, heat shock also activated ERK MAPKs in a potent (>60-fold), delayed (>30 min), and sustained (>120 min) manner. These characteristics suggested a novel mechanism of ERK MAPK activation and became the focus of this study. A MEK-specific inhibitor, PD98059, inhibited heat shock ERK MAPK activation by >75%. Surprisingly, a role for Ras in the heat shock response was eliminated by the failure of a dominant-negative RasAsn-17 mutant to inhibit ERK MAPK activation and the failure to observe increases in Ras-GTP. Heat shock also failed to stimulate activation of A-, B-, and c-Raf. Instead, a serine/threonine phosphatase inhibitor, okadaic acid, activated ERK MAPK in a similar manner to heat shock. Furthermore, pretreatment with suramin, generally recognized as a broad range inhibitor of growth factor receptors, inhibited both okadaic acid-stimulated and heat shock-stimulated ERK MAPK activity by >40%. Inhibiting ERK MAPK activation during heat shock with PD98059 enhanced losses in cell viability. These results demonstrate Ras- and Raf-independent ERK MAPK activation maintains cell viability following heat shock.

The cellular responses to a huge variety of external stimuli are mediated by sophisticated arrays of phosphorylation cascades. The extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) pathway was one of the first phosphorylation cascades to be well characterized following mitogenic stimulation by peptide growth factors (1). Specifically, the steps following the engagement of specific growth factor receptor at the plasma membrane include the activation of the small G-protein Ras, with Ras-GTP then directly interacting with and regulating the activation of the Ser/Thr kinase, Raf. Raf in turn phosphorylates the dual specificity kinase MEK (MAPK kinase), which then phosphorylates and activates the ERK MAPKs. This signaling cascade of Ras → Raf → MEK → ERK MAPKs has been implicated in the regulation of cellular differentiation, transformation, and proliferation through activation of intracellular substrates including transcription factors, such as Elk-1, c-Jun, and ATF2, and other protein kinases, including p90RSK (2).

Other subfamilies of Ser/Thr kinases, collectively called stress-activated MAPKs, have been characterized following their preferential activation in response to extracellular stress stimuli such as osmotic shock, UV light, oxidative stress, low pH, or heat shock (3). These stress-activated protein kinases, which include the c-Jun NH2-terminal kinase MAPKs (JNK MAPKs) and p38 MAPKs, act within phosphorylation cascades that show overall similarity in organization but are distinct from the ERK MAPK cascade (4). Thus the stress-activated protein kinases are regulated by distinct dual specificity kinases that are in turn regulated by specific upstream kinases. These kinase pathways are implicated in signaling apoptosis, cell differentiation, and transformation (4).

Although specific MAPKs function exclusively within their respective pathways, it has been recently shown that a single stimulus can activate two or more MAPK cascades to varying degrees (5–7). The regulation of a cellular response may therefore be the result of several signaling cascades working in concert. It is now clear that the ERK MAPKs can be activated by various stress stimuli including heat shock, hydrogen peroxide, arsenite, and osmotic shock (8–11). This dispels the earlier model of ERK MAPK activation exclusively by mitogenic stimuli (1).

It still remains to be clarified whether all forms of cellular stress and mitogenic stimuli activate identical signaling events to activate the ERK MAPKs. The increasing complexity of these signaling events revealed through recent research suggests that this is not the case. Although a number of studies have demonstrated that the classical pathway of Ras → Raf → MEK mediates ERK MAPK activation in response to oxidative stress and arsenite (10–12), the ERK MAPK activation following exposure to pervanadate was not mediated by this series of events (13). This has led to the suggestion that there may be MEK-independent routes to ERK MAPK activation when cells are exposed to different forms of stress (13).

In the current study, we have investigated heat shock-induced activation of the MAPK cascades in the IL3-dependent, murine pro-B cell line, BaF3. Surprisingly, we have now found...
that heat shock stimulated the phosphorylation and activation of the ERK MAPKs. Furthermore, the “stress-activated” JNK MAPKs but not the p38 MAPKs were phosphorylated and activated. The robust activation of ERK MAPK was unexpected. We focused on characterizing the delayed kinetics of ERK MAPK activation following heat shock. This suggested a novel stress-activated signaling pathway to ERK MAPKs which, in addition, was more potent than that elicited following the exposure to the cytokine IL3. We have demonstrated that heat shock activates ERK MAPKs via a pathway that involves MEK but is independent of Ras and Raf activation. We present evidence that suggests heat shock activates the ERK MAPKs, not through a sequence of upstream “on” signals but instead by deactivating a serine/threonine phosphatase-dependent “off” signal. The resulting activation of ERK MAPKs appears to play a role in maintaining cell viability following the heat shock insult.

**EXPERIMENTAL PROCEDURES**

**Materials**—The monoclonal antibody Ras R02120 was obtained from Transduction Laboratories, and the monoclonal antibody anti-phospho-activating domain residues from Upstate Biotechnology, Inc. Polyvalent antibodies that recognized ERK1 (C-16) and ERK2 (C-14) were from Santa Cruz Biotechnology. RPMI 1640 medium, heat-inactivated fetal calf serum, penicillin/streptomycin, glutamine, and phosphate-buffered saline were purchased from Life Technologies, Inc. PD98059, bisindolylmaleimide, and murine recombinant IL3 were obtained from Calbiochem, whereas suramin was from ICN Biomedicals Inc. Antibodies that recognize the total (phosphorylated and non-phosphorylated) forms of ERK1 (C-16, sc-93), ERK2 (C-14, sc-154), JNK1 (C-17, sc-474), p38 MAPK (C-20, sc-535), Raf, A-Raf, and B-Raf were from Santa Cruz Biotechnology. Phospho-specific p44/42 ERK MAPK (Thr202/Tyr204, number 9101S), phospho-specific p38 MAPK (Thr180/Tyr182, number 9211S), phospho-specific MEK1/2 (Ser217/Ser221, number 1211S), and total MEK (number 9122) were from New England Biolabs. [γ-32P]ATP was from PerkinElmer Life Sciences. The Lumi-Light Plus chemiluminescent substrate was from Roche Molecular Biochemicals. Molecular mass markers and Hyperfilm MP were from Amersham Pharmacia Biotech. Recombinant Elk-(307–428) and c-Jun-(1–135) were expressed as glutathione -transferase (GST) fusion proteins and purified by glutathione-Sepharose chromatography for use as substrates for ERK and JNK MAPK, respectively. All other reagents were from Sigma.

**Cell Culture and Treatment**—All cells were grown at 37 °C in a controlled atmosphere of 5% CO2, 95% air and 99% relative humidity. The IL3-dependent BaF3 cell line and BaF3-derived N6F-20 cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) sodium deoxycholate, 10% (v/v) glycerol, 25 mM NaF, 10 mM MgCl2, 1 mM EDTA, 1 mM NaVO4, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cells lysates (1 mg) were incubated with GST-Raf-binding domain of Raf (50 μg) for 120 min at 4 °C and then captured on 20 μl of glutathione-Sepharose (1:1 (v/v) slurry in Buffer B) for an additional 60 min. The pellets were then recovered and washed with Buffer B (3 × 200 μl). SDS-PAGE sample buffer (20 μl) was added and then Ras was detected by immunoblotting.

**Raf Activity Assay**—Activation of Ras was assayed by a linked MEK/ERK kinase assay utilizing MBP as substrate (19). Treated BaF3 cells were washed in phosphate-buffered saline and then lysed in Buffer C (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF freshly supplemented with 1% (v/v) Triton X-100, 0.1% (w/v) BSA, 20 μg/ml aprotinin, 1 μg/ml benzamidemethyl sulfonyl fluoride, 2 mM NaVO4, and 2.5 mM MgCl2, 0.1% (v/v) β-mercaptoethanol, 0.3% (v/v) Brij-35, 6.5 μg/ml GST-MEK, 100 μg/ml GST-ERK2, 10 mM MgCl2, and 0.2 mM ATP). The reaction was terminated by adding 10 μl of supernatant to 40 μl of a 1:1 (v/v) slurry in Buffer C for a further 2 h. The pellets were washed extensively in Buffer C and resuspended in Buffer D (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.5% (w/v) BSA, 20 μg/ml leupeptin, 1 μg/ml benzamidemethyl sulfonyl fluoride, and 2 mM NaVO4). The MEK/ERK kinase assay was performed as described (1) with 2.25 μl of [γ-32P]ATP per assay. The kinase reaction was allowed to proceed for 30 min at 30 °C and then the MBP phosphorylation reaction was terminated by spotting onto P81 paper. The paper was washed extensively in 75 mM phosphoric acid, and incorporated radioactivity was quantitated by Cerenkov and then the MBP phosphorylation reaction was terminated by spotting onto P81 paper. The paper was washed extensively in 75 mM phosphoric acid, and incorporated radioactivity was quantitated by Cerenkov counting. An equivalent volume of Buffer C replaced the antibody to serve as a control for each assay.

**Immunoblotting**—Proteins in cell lysates were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. For MAPK or Ras detection, membranes were blocked with 5% (w/v) milk powder in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 1% (v/v) Tween 20) and then blotted with primary antibodies diluted 1/250 to 1/1000, depending on the antibody and determined experimentally) in the block solution. Horseradish peroxidase-linked secondary antibodies were incubated 1/2000 in 1% (v/v) non-fat milk powder in TBST. The membranes were exposed to phosphotyrosine with the membranes blocked with 1% (w/v) BSA in TBST followed by incubation with the 4G10 primary antibody diluted 1/2000 in TBST and then the secondary antibody diluted 1/20,000 in 0.2% (w/v) BSA in TBST. In all cases, final detection was with Lumi-Light Plus chemiluminescent substrates according to the manufacturer’s instructions.
Heat Shock Stimulation of MAPK Phosphorylation—We treated BaF3 cells (2 × 10⁶) with IL3 (20 ng/ml, 10 min), hydrogen peroxide (H₂O₂, 0.5 mM, 10 min), phorbol ester (TPA, 1 μM, 5 min), osmotic shock (sorbitol, 0.5 M, 10 min), heat shock (42°C, 60 or 120 min), or left untreated as a control (Con). Lysates were analyzed by Western blotting with antibodies for either ERK MAPK (A), p38 MAPK (B), or JNK MAPK (C). The upper panel in each case shows results obtained with phospho-specific antibodies, and the lower panel shows that the total expression of each MAPK did not change significantly during the course of the experiment and therefore provides a control for protein loading. The molecular mass markers (kDa) are shown to the right of the panel. These experiments were repeated twice with comparable results.

**RESULTS**

**Heat Shock Stimulation of MAPK Phosphorylation**—We were interested in understanding the regulation of the ERK MAPK pathway by diverse extracellular stimuli including cytokines and stress stimuli. We used the IL3-dependent proB cell line, BaF3, to investigate activation of MAPKs by a range of different stimuli including heat shock. First, we treated IL3-deprived BaF3 cells with either the cytokine IL3 (20 ng/ml, 10 min), oxidative stress (0.5 mM hydrogen peroxide, 10 min), the phorbol ester TPA (1 μM, 5 min), osmotic shock (0.5 M sorbitol, 10 min), or heat shock (42°C, 60 or 120 min), and we determined the phosphorylation of three major subfamilies of MAPKs by immunoblotting with antibodies specific for the phosphorylation of each of these subfamilies. As shown in Fig. 1A, IL3, TPA, and heat shock stimulated the phosphorylation of the ERK MAPKs. This stimulation by heat shock exceeded that of the other stress stimuli and was more akin to stimulation by the mitogenic factor, IL3, or the phorbol ester, TPA. This suggests that heat shock, unlike these other stress stimuli, can potentially activate the ERK MAPK pathway in BaF3 cells.

In contrast, the two stress stimuli of hydrogen peroxide and sorbitol stimulated phosphorylation of p38 MAPK, whereas heat shock could not effectively increase the phosphorylation of these MAPKs at the times tested (Fig. 1B). In addition, the phosphorylation of the JNK MAPKs was potently stimulated by sorbitol or heat shock and moderately stimulated by hydrogen peroxide (Fig. 1C). Upon longer exposures of these immunoblots, phosphorylation of JNK MAPKs could be observed following IL3 exposure as previously reported (20). To confirm the activation of JNK MAPKs by heat shock and IL3, we also measured JNK MAPK activity using a c-Jun pull-down assay (17). Whereas JNK MAPK activation was rapid and transient as described previously (14), the kinetics of JNK MAPK activation by heat shock showed a peak of activation following heat shock for 60 min and a decline at the 120-min time point. This is in agreement with the kinetics of heat shock-stimulated JNK MAPK phosphorylation shown in Fig. 1C.

These combined results confirm that each MAPK subfamily shows distinct differences in phosphorylation in response to extracellular stimuli. Our surprising result was that elevated temperature was a potent stimulus for ERK MAPKs in addition to the JNK MAPKs that have been traditionally classed as stress-activated protein kinases. With the unusual kinetics of robust ERK MAPK activation observed following the heat shock stimulus, we then focused on how the ERK MAPK pathway could be regulated by this stress. Furthermore, the availability of a specific chemical inhibitor of the ERK MAPK pathway, PD98059 (21), would allow us to evaluate the cellular consequences of ERK MAPK activation under these circumstances.

**Heat Shock Stimulates a Strong, Sustained but Delayed Activation of ERK MAPK**—We were interested in further evaluating the events leading to phosphorylation of the ERK MAPKs following the heat shock stimulus. ERK MAPK activity in soluble extracts was assayed in a pull-down kinase assay using GST-Elk-(307–428) as a substrate (16). As shown in Fig. 2, negligible ERK MAPK activity was observed during the first 10–30 min exposure to 42°C heat shock. However, activation of ERK MAPKs accompanying heat shock for 60 and 120 min was far greater than that achieved with a 10-min exposure to the cytokine stimulus, IL3. Cerenkov counting quantitatively the extent of ³²P incorporation, and data from three independent experiments indicated that 42°C heat shock for 60 min activated ERK MAPK 60-fold compared with 32-fold activation by IL3 (20 ng/ml) (Fig. 2A). The ERK MAPK activation by heat shock was further increased during the 2nd hour of heat shock (Fig. 2, A and B). This contrasts with previous results of rapid and transient activation of ERK MAPKs by cytokines such as IL3 or granulocyte colony-stimulating factor (14). Furthermore, it differs from the JNK MAPK activation we have noted in the heat-shocked BaF3 cells, which is maximal at 60 min and declining in activity and phosphorylation by 120 min (Fig. 1C and Footnote 2).

The potent, sustained but delayed activation of ERK MAPKs by heat shock suggested that the pathway initiated by this cellular stress may differ from that utilized by the cytokine IL3. We therefore set out to examine which of the steps involved in the classic ERK MAPK pathway (as utilized by IL3) were the targets for activation by heat shock. Starting with the most global changes normally associated with growth factor and cytokine signaling, we examined the effects of these stimuli on...
Heat Shock Activation of the ERK MAPK Pathway

**FIG. 2.** Comparison of ERK MAPK activation by heat shock and IL3. BaF3 cells (2 x 10^6) were cytokine-starved (3 h) before treatment with IL3 (20 ng/ml, 10 min), heat shock (42 °C for the indicated times), or left untreated as a control (Con). Detergent-soluble lysates were prepared, and ERK MAPK activity was determined by pulldown kinase assay with GST-Elk (207–428) as substrate. A pulldown kinase reaction without cell lysate was also included as a control for the procedure (no lysate). Autoradiography indicated the incorporation of [gamma-32P]ATP into the GST-Elk substrate (upper panel). Coomassie staining confirmed equal loading of GST-Elk protein (lower panel). B, time course of heat shock-stimulated ERK MAPK activity. Cerenkov counting of phosphorylated GST-Elk was used to quantitate the ERK MAPK activity (cpm) in heat-shocked or IL3-treated BaF3 cells. Values represent means ± S.D. for four independent experiments.

The next step in identifying the signaling events involved in the heat shock response, we exposed BaF3 cells to heat shock in the continued presence of IL3 (i.e., "unstarved" BaF3 cells). In this way, the IL3-responsive pathways would remain desensitized through their continuous exposure to IL3. Under these conditions, heat shock was still capable of activating the ERK MAPKs to approximately the same extent. We therefore conclude a pathway independent of IL3 receptor activation mediates the activation of ERK MAPKs in response to prolonged heat shock.

**PD98059 Inhibits ERK MAPK Activation and Phosphorylation**—It has been widely demonstrated that MEK1 and -2 are the highly conserved upstream kinases involved in the stimulation of the ERK MAPK cascade by various mitogenic and stress stimuli (2). The protein kinase inhibitor PD98059 has been used extensively to implicate MEK1/2 in regulating the ERK MAPKs (21). As shown in Fig. 4, we tested the ability of PD98059 to inhibit ERK MAPK activation by IL3 or heat shock. We chose two concentrations of PD98059 (10 and 30 μM) that lie within the range that maintains the specificity for MEK1/2 inhibition (21). We found that control, IL3, or heat shock-stimulated levels of ERK MAPK activity were inhibited by at least 40% by 10 μM PD98059 (Fig. 4A), and the level of inhibition increased to at least 50% for 30 μM (Fig. 4B). In both cases, the level of inhibition achieved by PD98059 was greatest for heat-shocked cells. In all cases, controls with appropriate volumes of Me2SO were included to account for the fact that stock solutions of PD98059 had been prepared in Me2SO (Fig. 4, A–D).

We confirmed that 30 μM PD98059 abrogated heat shock and IL3-stimulated phosphorylation of ERK1 and ERK2 (Fig. 4C, upper panel), while not altering the total levels of expression of the ERK MAPKs (Fig. 4C, lower panel). In contrast, we confirmed that the activation of JNK MAPKs by IL3 or heat shock was not inhibited by 30 μM PD98059 (Fig. 4D, upper panel), and under these conditions the total levels of expression of JNK MAPKs did not change (Fig. 4D, lower panel). This is in agreement with previous studies showing that the activation of JNK MAPKs is not inhibited by PD98059 treatment (21).

The involvement of MEK was further confirmed by the observation of increased phosphorylation of MEK by immunoblotting cell extracts prepared from heat-shocked cells with antibodies recognizing the phosphorylated, activated form of MEK1 and -2. These results confirm that the involvement of MEK upstream is conserved in regulating heat shock-stimulated ERK MAPK phosphorylation and activity.

**Heat Shock Stimulates a Ras-independent Pathway to Activate ERK MAPK**—The next step in identifying the signaling route to ERK MAPK activation by heat shock entailed the identification of the potential candidates for membrane proximal signaling events. The small G-protein, Ras, has been implicated in the regulation of ERK MAPK activity stimulated by...
Heat Shock Activation of the ERK MAPK Pathway

Various growth factor receptors (23). In addition, recent studies have demonstrated that Ras-dependent signaling pathways are stimulated by oxidative stress elicited by hydrogen peroxide (12). The role of Ras in heat shock signaling was examined by investigating the effects of a dominant-negative mutant of Ras, RasAsn-17 (14, 15, 20). In the BaF3 cell line this has been facilitated by the stable introduction of an expression plasmid that permits the inducible expression of RasAsn-17 when cells are cultured overnight in the presence of IPTG (14, 15, 20). This system has successfully demonstrated that Ras is essential in the IL3-induced activation of c-Raf, ERK MAPKs, and JNK MAPKs (15, 20) but that these Ras-dependent pathways may not be essential in the IL3 stimulation of proliferation (15).

We induced expression of RasAsn-17 in the N6F-20 BaF3 cell line by overnight incubation with IPTG (14). As demonstrated in Fig. 5A, although this induction of RasAsn-17 significantly attenuated the ERK MAPK response to IL3 (ANOVA, p < 0.05), heat shock-stimulated ERK MAPK activity at 60 or 120 min remained uninhibited. We confirmed that the levels of RasAsn-17 remained elevated during the heat shock protocol by immunoblotting cell lysates for expression of total Ras protein (Fig. 5B, upper panel). Reprobing the immunoblot with the ERK MAPK 1/2 antibody confirmed that the expression of ERK MAPKs remained constant and there was equivalent ERK MAPK loading in all lanes (Fig. 5B, lower panel). Thus, the failure of RasAsn-17 to inhibit ERK MAPKs under these heat shock conditions could not be attributed to instability of the RasAsn-17 protein at 42 °C. We could not further evaluate the effectiveness of the RasAsn-17 following heat shock (for example by ensuring that the RasAsn-17 expressed in the heat-shocked cells could inhibit subsequent IL3 activation of ERK MAPKs) because the heat shock activation of ERK MAPKs remained sustained for more than 2 h following heat shock (see Fig. 5C). Therefore, this would interfere with any ability to detect a comparatively smaller activation of ERK MAPKs by IL3 even after a 2-h recovery period. Furthermore, during any longer recovery periods we might anticipate the de novo synthesis of RasAsn-17 molecules, and this would interfere with interpretation of these results.

Because recent evidence suggests that RasAsn-17 may not effectively inhibit all mechanisms of Ras activation (24), we sought further evidence to confirm or refute the involvement of Ras in the activation of ERK MAPKs by measuring Ras activation. This ARIA utilizes the Ras-binding domain of Raf to isolate active Ras (i.e. Ras-GTP) (18). A time course of IL3 exposure revealed that maximal Ras activation occurs within 2 min (Fig. 6A). Activation of both Ras and ERK MAPKs by IL3 was therefore rapid and transient (15). However, when Ras-GTP levels in heat shock-stimulated cells were assessed, we observed these were not consistently greater than those observed in control cells (Fig. 6B). We acknowledge that it is possible that any heat shock elevation of Ras-GTP was transient and missed in our time course. However, transient Ras

![Fig. 4](https://example.com/fig4.png)  
**Fig. 4.** PD98059 attenuates ERK MAPK activation by either IL3 or heat shock. Cytokine-starved BaF3 cells (2 x 10^5) were pretreated with PD98059 at a final concentration of either 10 μM (A) or 30 μM (B) during the final hour of the 3-h cytokine starvation period. For control cells, exposure to the appropriate volume of Me2SO vehicle was included during this final hour. Cells were then treated with IL3 (20 ng/ml, 10 min), heat shock (42 °C, 60 min), or left untreated as a control. Lysates were analyzed for ERK MAPK activation using the GST-Elk pulldown kinase assay. The activity of the cells pretreated with the appropriate concentration of Me2SO was defined as 100%, and the other activities were calculated as a percentage of maximum stimulation. The data presented are means ± S.D. (n = 3 independent observations). In all treatments, * indicates a significant inhibition of ERK MAPK activity by PD98059 (p < 0.05, one-way analysis of variance test with Fischer’s Least Significant Difference test) when compared with the controls preincubated in the presence of Me2SO alone. C, BaF3 cells (2 x 10^5) were treated exactly as described and then analyzed by immunoblotting for either phosphorylated ERK1/2 (upper panel) or total ERK1/2 MAPKs (lower panel). This experiment was performed twice with comparable results. D, an independent set of cells, pretreated with PD98059 (30 μM), was stimulated identically as described. Lysates were analyzed for JNK activation using the GST-Jun pulldown kinase assay. A sample in which cell lysate was replaced with hysis buffer only was used as a negative control. Autoradiography of GST-Jun resolved by SDS-PAGE indicated incorporation of 32P from [γ-32P]ATP into GST-Jun. Coomassie staining confirmed equal loading of GST-Jun protein (lower panel). This experiment was performed twice with comparable results.
activation is most often associated with transient ERK MAPK activation (15). The inability of Ras\textsuperscript{Asn-17} to block heat shock-stimulated ERK MAPK activity, coupled with the finding of negligible increases in Ras-GTP levels during heat shock, indicates that the pathway utilized by heat shock to activate ERK MAPK is most likely one that is independent of Ras. In the BaF3 cell line, pathways other than the well characterized Ras/ERK MAPK pathway must play a role in the response to heat shock.

Heat Shock Activation of ERK MAPKs Is Independent of Raf—The activation of ERK MAPKs is not exclusively mediated by a Ras-dependent pathway in all cell types. It has been documented that PKC can directly phosphorylate serine residues of Raf to modulate its kinase activity (25). We therefore sought to investigate the kinase activity of Raf stimulated by heat shock and determine its role in regulating the ERK MAPKs. There are three Raf isoforms as follows: c-Raf (which is also known as Raf-1), A-Raf, and B-Raf. The activity of each individual Raf isoform in response to heat shock and IL3 was measured by a MEK/ERK-linked Raf kinase assay as described under “Experimental Procedures.” As shown in Fig. 7, a 2-min exposure to IL3 stimulated substantial Raf activity over control. Treatment of BaF3 cells with the cytokine stimulated an 18-, 5.5-, and 2.4-fold activation over control of c-Raf, A-Raf, and B-Raf kinase activity, respectively. The relatively lower fold activation stimulated by IL3 in B-Raf was due to a higher degree of constitutive basal activity, and this is consistent with previously published results with B-Raf assays (26). In contrast, 42 °C heat shock for 15, 30, 45, and 60 min failed to stimulate comparable activity from any of the three Raf isoforms (Fig. 7, A–C). It could be argued that we may have missed a transient Raf activation due to our chosen time points, but this is unlikely when we would expect sustained Raf activity to regulate sustained heat shock-induced ERK MAPK activity as has been previously demonstrated (19).

An involvement of PKC was also discounted when we found that the PKC-specific inhibitor, bisindolylmaleimide I (10 μM), failed to consistently attenuate heat shock-induced ERK MAPK activity. The inter-batch variation observed with bisindolylmaleimide I prompted investigation using an inactive form of this compound, bisindolylmaleimide V, as well as chronic exposure to phorbol esters to down-regulate expression of phorbol ester-sensitive PKC isoforms. Again, the results with bisindolylmaleimide were inconsistent. However, we consistently observed that down-regulation of PKC with prolonged overnight exposure to TPA (1 μM) failed to attenuate the ERK response (data not shown). Taken together these results indicate that heat shock-induced MEK and ERK MAPK activity does not involve upstream regulation by the three Raf isoforms or the phorbol ester-sensitive PKC isoforms.

Role of Growth Factors Receptors in Regulating the Heat Shock-stimulated ERK MAPK Activity—Several reports have implicated growth factors and their receptors as mediators in the response to extracellular stress. In these models, stress acts to stimulate release of growth factors, which then act through an autocrine/paracrine mechanism to activate the traditional Ras → Raf → MEK → ERK MAPK cascade (11, 12). Although we have already shown that heat shock does not activate ERK
MAPKs through the traditional cascade involving Ras or Raf, our observation of delayed kinetics of heat shock-stimulated ERK MAPK activation may suggest the involvement of an autocrine signaling loop. As the activation of the ERK MAPK signaling pathway by peptide growth factors and cytokines is mediated by the hetero- or homo-multimerization of ligand-specific receptors localized at the plasma membrane, inhibiting these events at the membrane may effectively abrogate signaling. We tested the involvement of growth factor receptors in heat shock signaling with the use of suramin. This analog of heparin sulfate has been implicated as an inhibitor of a heterogeneous population of growth factor and cytokine receptors and has been shown to inhibit ERK MAPK activation by serum, epidermal growth factor, platelet-derived growth factor, tumor necrosis factor-α, as well as ERK MAPK activation by oxidative stress elicited by hydrogen peroxide and arsenite (11, 12, 27, 28).

Fig. 8A represents a typical experiment where pretreatment of cells with 300 μM suramin abrogated ERK MAPK activity stimulated by IL3. This concentration of suramin also inhibited ERK MAPK activation following heat shock for 60 min by 80% (ANOVA, p < 0.05), but it failed to significantly inhibit ERK MAPK activity stimulated by heat shock for 120 min (ANOVA, p > 0.05) (Fig. 7B). We tested whether this failure of suramin to inhibit the 120-min heat shock response could be due to different mechanisms of signaling at the two time points or be due to problems of stability of suramin under these incubation conditions. Preheating of 300 μM suramin in starvation medium at 42 °C for 120 min prevented its ability to subsequently abrogate IL3-stimulated ERK MAPK activity (ANOVA, p < 0.05) (Fig. 8B). Thus suramin was not stable for 120 min under these conditions at 42 °C, and we cannot draw any conclusions on whether the mechanisms involved in ERK MAPK activation at 60 and 120 min of heat shock differ.

We sought further support for a role of released growth factors mediating the heat shock ERK MAPK response. We tested whether ERK MAPKs would remain activated following removal from the elevated temperatures. Our results in Fig. 8C demonstrate that heat shock-stimulated (42 °C, 60–120 min) ERK MAPK activity remains elevated after a 60-min recovery period at 37 °C. Thus ERK MAPK activation by the 60-min heat shock followed by 60 min of recovery was similar to the activation observed following 120 min of heat shock. This may suggest the presence of extracellular signaling molecules released during the heat shock treatment. Next, we tested the effects of media conditioned during the heat shock of these cells. However, this conditioned media failed to activate ERK MAPK activity when applied to starved BaF3 cells. Finally, we attempted to prevent ERK MAPK activation with the use of a protein synthesis inhibitor, cycloheximide. We found that the heat shock activation of ERK MAPKs was not inhibited by preincubation with 10 μM cycloheximide. The combined results from these studies suggest that, although growth factor receptors may be involved in the heat shock response, they may be directly modulated during heat shock without requirement of release of autocrine/paracrine factors.

Exposure to the Phosphatase Inhibitor Okadaic Acid Also

Fig. 7. Heat shock fails to activate Raf isoforms. Cytokine-starved BaF3 cells (2 × 10^7) were stimulated with IL3 (20 ng/ml, 2 min), left untreated as a control (Con), or heat-shocked (42 °C) for the indicated times. A detergent-soluble protein extract was made and used to isolate c-Raf (A), A-Raf (B), or B-Raf (C) by immunoprecipitation with Raf isoform-specific antibodies. Kinase activity was then subsequently measured by a MEK/ERK-linked Raf assay as described under “Experimental Procedures.” The Raf activity in control cells was set at 1, and the stimulated Raf activity expressed as fold activation over untreated cells (Con).
Stimulates Delayed ERK MAPK Activation—An alternative mechanism to account for a delayed and sustained activation of a protein kinase could involve stress-induced inhibition of a protein phosphatase. Whereas inhibition of tyrosine phosphatases by oxidative stress is a well recognized modulator of MAPK pathways (29, 30), in the situation of heat-shocked BaF3 cells the lack of substantial changes in tyrosine phosphorylation together with modulation of the pathway at the level of MEK suggests that serine/threonine phosphatases may be more likely candidates for targets. To evaluate this hypothesis we exposed BaF3 cells to 1 mM okadaic acid (31–34) for up to 2 h. We did not observe activation of ERK MAPKs following the short term exposure (10 min) to okadaic acid,2 and this is consistent with a delayed activation of ERK MAPKs we have previously observed upon okadaic acid exposure of cardiac myocytes (33). At 60 and 120 min of exposure to okadaic acid, we noted at least 9.5-fold activation of ERK MAPKs (Fig. 9A) and increased phosphorylation of MEK (Fig. 9B).

![Fig. 8](image8.png)

**Fig. 8.** Effect of suramin on heat shock-stimulated ERK MAPK activity. Cytokine-starved BaF3 cells (2 × 10⁶) were pretreated with suramin (300 μM) for 60 min prior to treatment with IL3 (20 ng/ml, 10 min), heat shock (42 °C for 60 min or 120 min, HS60 and HS120, respectively), or left unstimulated as a control (Con). A set of cells was treated identically in the absence of suramin for comparison (−Suramin). To test the stability of suramin under these incubation conditions, suramin, or an equivalent volume of media, was also preincubated at 42 °C (IL3c) in the absence of cells before testing its effects on IL3-stimulated ERK MAPK activation. A, ERK MAPK activity was measured by GST-Elk pulldown kinase assay as described under “Experimental Procedures.” Autoradiography of GST-Elk indicated the incorporation of 32P from [γ-32P]ATP into the substrate (upper panel). Coomassie staining confirmed equal loading of GST-Elk protein (lower panel). B, the activity of ERK MAPK was determined by Cerenkov counting of GST-Elk. Results are expressed as fold activation over control levels and are expressed as means ± S.D. (n = 3 independent observations). In all treatments, * indicates a significant inhibition of ERK MAPK activity by suramin (p < 0.05, one-way analysis of variance test with Fischer’s Least Significant Difference test) when compared with controls. C, cytokine-starved BaF3 cells (2 × 10⁶) were treated with heat shock (42 °C) for either 60 or 120 min and then transferred to 37 °C for a further 60 min of recovery. Detergent-soluble cell lysates were then prepared, and ERK MAPK activity was measured by pulldown kinase assay as described. Values represent mean ± S.E. from three independent experiments.

Stimulates Delayed ERK MAPK Activation—An alternative mechanism to account for a delayed and sustained activation of a protein kinase could involve stress-induced inhibition of a protein phosphatase. Whereas inhibition of tyrosine phosphatases by oxidative stress is a well recognized modulator of MAPK pathways (29, 30), in the situation of heat-shocked BaF3 cells the lack of substantial changes in tyrosine phosphorylation together with modulation of the pathway at the level of MEK suggests that serine/threonine phosphatases may be more likely candidates for targets. To evaluate this hypothesis we exposed BaF3 cells to 1 mM okadaic acid (31–34) for up to 2 h. We did not observe activation of ERK MAPKs following the short term exposure (10 min) to okadaic acid, and this is consistent with a delayed activation of ERK MAPKs we have previously observed upon okadaic acid exposure of cardiac myocytes (33). At 60 and 120 min of exposure to okadaic acid, we noted at least 9.5-fold activation of ERK MAPKs (Fig. 9A) and increased phosphorylation of MEK (Fig. 9B). Although the level...
of ERK MAPK activation following okadaic exposure was not as potent as heat shock stimulation of these kinases, it was noted that suramin was also effective at abrogating the effects of both okadaic acid and heat shock (Fig. 9). Suramin (300 μM) inhibited the ERK activity stimulated by 120 min of treatment with okadaic acid or 60 min of heat shock (ANOVA, p < 0.05) (Fig. 9). Although the statistical analysis did not indicate a significant difference for the effects of suramin during 60 min of okadaic acid treatment, there was a trend toward lower ERK activation in the presence of suramin (Fig. 9). These results suggest that the results of experiments using suramin should be treated with caution because it is currently unlikely that effects of okadaic acid are mediated by secondary events of growth factor release and/or growth factor receptor signaling. Instead, we would suggest that delayed ERK MAPK activation mediated by either heat shock or okadaic acid is a result of an attenuated control of negative regulators of the protein kinases MEK and/or ERK MAPKs.

Heat Shock-stimulated ERK MAPK Activity Mediates Cell Viability following Stress Insult—It has been suggested that the activation of the ERK MAPK cascade in response to stress is involved in a concerted defense mechanism to protect the cells (10, 12). The ability of BaF3 cells to survive heat shock was therefore measured by trypan blue staining. Attenuation of ERK MAPK activity by the MEK inhibitor PD98059 (30 μM) was used to investigate the role of heat shock-stimulated ERK MAPK in maintaining cell viability. We measured cell viability directly after a 1-h exposure to heat shock or 24 h after this treatment (Fig. 10). There was no significant difference in the number of viable cells immediately after heat shock in the presence or absence of the MEK inhibitor PD98059 (Fig. 10A). From this result, we would suggest that ERK MAPK activation is not critical for the short term survival in these cells.

After a 24-h period at 37 °C, the number of viable cells under control conditions had decreased from 98 to 86% (Fig. 10B). In the presence of PD98059, this percentage of viable cells decreased from 95 to 76% (ANOVA p > 0.05). The presence of PD98059 therefore did not appear to significantly affect survival of control cells, although this result will require further investigation. When we examined the effects of heat shock, we observed that the viability of heat-shocked cells had decreased to 40% after 24 h of further culture at 37 °C. Attenuation of ERK activity by PD98059 resulted in a further decrease of viability to 26% which was significantly different (ANOVA p < 0.05) to viability of heat-shocked cells in the absence of the inhibitor (Fig. 10B). These results indicate that an attenuation of ERK MAPK activity by PD98059 is accompanied by increased loss of viability in the BaF3 cell population. This implicates ERK MAPKs in maintaining the viability of BaF3 cells following stress from heat shock.

**DISCUSSION**

It is now recognized that both the extent and kinetics of ERK MAPK activation are critical determinants of the cellular response to growth factors and cytokines (35). Transient ERK MAPK activation is most commonly associated with cellular proliferation, whereas prolonged ERK MAPK has been associated with nuclear translocation of these kinases and cellular differentiation (36). Presumably the translocation of ERK MAPKs to the nucleus following prolonged activation permits access to different subsets of transcription factor substrates essential for the differentiation process (35).

There is now increasing evidence that activation of the ERK MAPKs can also be stimulated by a variety of stress stimuli. In most cases, ERK MAPK activation in response to these stresses is transient. For example, ERK MAPK activation by hydrogen peroxide, arsenite, or osmotic stress is maximal within 10–20 min and rapidly returns to basal levels within the 1st hour of exposure (11, 12, 37, 38). Heat shock has been well documented as a stress that initiates long term changes within cells. The best characterized of these changes is the stimulation of heat shock protein synthesis in a protective response to prevent further cell damage (39, 40). Indeed, heat shock has been proposed as an experimental method for enhancing heat shock protein synthesis to protect against further cell insults such as ischemia (39). Few studies have considered earlier events in the detection of heat shock by cells or whether heat shock also activates cytoplasmic protein kinase signaling cascades. In a number of cell types, heat shock apparently fails to activate ERK MAPKs but does activate the stress-activated MAPKs such as the JNK MAPKs (3, 38). However, these responses are apparently cell type-dependent, and heat shock prevents activation of JNK MAPKs and p38 MAPKs in U937 cells but does activate ERK MAPKs in H56 hepatoma cells, NIH 3T3 fibroblasts, human HeLa cells, human KB carcinoma cells, or during whole animal hyperthermia (41–45). In all of these examples, the activation of ERK MAPK was not examined beyond an initial 60 min of heat shock, and so any long term effect on signaling pathways was not evaluated.
In the present study, we have demonstrated that ERK and JNK MAPKs, but not p38 MAPKs, are activated by 42 °C heat shock at 60 and 120 min (Fig. 1). In the heat-shocked BaF3 cells, we observed maximal phosphorylation and activation of JNK MAPKs at 60 min. Due to the lack of a specific chemical inhibitor of the JNK MAPKs and their complex pathways of upstream regulators, the elucidation of the mechanisms underlying this activation and its subsequent cellular effects would prove difficult. Furthermore, the activation of JNK MAPKs by heat shock was first reported when these protein kinases were cloned (3) and has been subsequently shown in numerous studies (for recent examples see Refs. 46–48). Therefore, we further examined the activation of ERK MAPKs by heat shock, concentrating on the mechanism of activation and the possible downstream events regulated by this signal transduction pathway.

We characterized the kinetics of ERK MAPK activation in the IL3-dependent proB cell line BaF3 as sustained at maximal levels over a 2-h period (Fig. 2). We have observed similar results in the IL3-dependent myeloid cell line NFS-60.2 Activation was also delayed (Fig. 2), and this suggested that in these cells ERK MAPK is activated either by a novel pathway or by the classical MAPK pathway following the release of growth factors and/or cytokines.

Apart from these important aspects of the long duration and delayed nature of ERK MAPK activation by heat shock in our system, it was also apparent that the level of ERK MAPK activation exceeded that stimulated by the cytokine IL3 (Fig. 2). IL3 is a critical requirement for the proliferation and survival of these cells. We were therefore interested in identifying the upstream events stimulated by heat shock that potentially activate the ERK MAPKs. Specifically we evaluated whether heat shock stimulated the well characterized Ras → Raf → MEK → ERK MAPK cascade or whether a more potent alternative pathway to ERK MAPK activation may be recruited when these cells were stressed by exposure to elevated temperatures. An alternative pathway could provide a rational approach to designing alternative ways to stimulate ERK MAPKs in these cells, thus altering cell proliferation and/or survival, independent of the effects of IL3.

MEK was implicated in heat shock signaling in the BaF3 cells when we showed that the MEK-specific inhibitor, PD98059. Inhibited heat shock-stimulated ERK activity (Fig. 4). The specificity of this inhibitor has been documented (21), and it is now widely used in evaluating the functional consequences of ERK MAPK activation. Furthermore, heat shock stimulated phosphorylation of MEK2 However, the current study also demonstrated that a dominant-negative mutant of Ras (Ras°) failed to attenuate heat shock stimulation of ERK MAPK activity (Fig. 5). This was confirmed when we demonstrated that Ras-GTP levels were not elevated during heat shock (Fig. 6). These data support a model in which a Ras-independent signaling pathway leads to potent ERK MAPK activation under these stress conditions.

It has been previously reported that the Raf/MEK/ERK cascade can be regulated independently of Ras through PKC directly regulating Raf through serine phosphorylation (49). Although this Ras-independent, PKC-dependent mechanism has only been infrequently reported to follow stimulation with a stress factor (50, 51), the role of PKC was nevertheless investigated. We have subsequently eliminated participation of PKC in heat shock signaling when a PKC-specific chemical inhibitor, bisindolylmaleimide 1, and down-regulation of phorbol ester-sensitive PKC isoforms upon prolonged TPA exposure failed to attenuate consistently heat shock-stimulated ERK MAPK activity (data not shown). More surprisingly, additional analysis of Raf activation revealed that none of the three Raf isoforms (Raf-1, A-Raf, and B-Raf) were activated during the 1st hour of heat shock (Fig. 7).

We have further established that heat shock-stimulated ERK MAPK activity does not involve an autocrine release of growth factors or cytokines. There is some evidence that supports cytokine induction following whole body heat shock (52). Furthermore, there is evidence that the activation of ERK MAPKs in response to stress may be mediated by an autocrine release of growth factors or cytokines then acting via their specific receptors to stimulate the classical Ras-dependent ERK MAPK pathway (11, 12). Most of this evidence comes from the use of suramin that has been demonstrated to bind to heparin-binding growth factor receptors such as the epidermal growth factor receptor, fibroblast growth factor receptor, and platelet-derived growth factor receptor (28, 53). To examine whether growth factor receptors may be involved in our system, we exposed BaF3 cells to suramin before and during the heat shock treatment. We found that suramin attenuated the heat shock activation of ERK MAPKs (Fig. 8, A and B). We sought further support for a role of growth factors, and we noted that heat shock-stimulated ERK MAPK activity remained sustained following a 60-min recovery period (Fig. 8C). This supported the idea that heat-shocked BaF3 cells were producing secondary signaling molecules, which would remain to stimulate the pathway even after the removal of the heat shock stimulus.

When we continued investigation of the involvement of growth factors, we found that medium conditioned in the presence of heat-shocked cells failed to stimulate ERK MAPK activity.2 This indicated that any autocrine growth factors were not released into media. This may be possible when cell surface-binding proteoglycans, such as heparin sulfate, bind growth factors and act as growth factor reservoirs to facilitate ligand receptor interaction at the cell surface (53). Alternatively, heat shock may stimulate ERK MAPK activity through a more direct influence on the growth factor receptors. The physical state of the cell membrane has been recognized to change in response to stress such as increased temperature, and this may modulate membrane-associated events such as receptor activation (54). In support of this general notion, the exposure of cells to ultraviolet light and osmotic stress perturbs the cell surface and alters the conformation of a number of different receptors leading to their clustering, activation, and subsequent alterations in MAPK signaling (55). Similarly, the activation of the platelet-derived growth factor-α receptor following mechanical stress of vascular smooth muscle cells occurs by perturbation of the receptor without engagement of the platelet-derived growth factor-binding site of the receptor (56). However, our failure to observe enhanced tyrosine phosphorylation following heat shock (Fig. 3) also opens the question of whether a growth factor-mediated pathway is in operation.

More recently there have been reports of ERK activity being regulated by mechanisms other than the traditional Raf → MEK → ERK MAPK cascade. Merin et al. (46) described the activation of JNK MAPKs by heat shock through a decreased rate of protein dephosphorylation (46). In this model, the basal activity of the MAPKs and MEK of non-stimulated cells is controlled by various serine/threonine phosphatases such as PP1 and PP2A. If the phosphatases are inhibited in any way, phosphorylation of their substrates will be favored. We have shown that okadaic acid, an inhibitor of the serine/threonine kinases PP1 and PP2A, stimulated ERK activity and MEK phosphorylation with delayed kinetics which resembled the activation following exposure to heat shock (Fig. 9). Surprisingly, the stimulation of MEK and ERK MAPK by okadaic acid was also sensitive to inhibition by suramin (Fig. 9), presumably...
revealing another nonspecific action of suramin in addition to its effects on a number of important intracellular signal transduction regulators including protein-tyrosine phosphatases, PKC, Cdc2, phosphatidylinositol 3′-kinase (see Ref. 58 and references therein). We propose that heat shock may activate MAPKs in the novel mechanism of inhibiting serine/threonine phosphatases.

The potent activation of ERK MAPK prompted our investigation into the physiological role that potent activation of ERK MAPK may play in heat shock signaling. We should emphasize, however, that we have not discounted a role for JNK MAPKs in the response to heat shock, but rather we have chosen to focus on the effects that the potentially-protective ERK MAPK pathway may play by exploiting the use of the specific MEK inhibitor PD98059 (21). Heat shock is synonymous with the universal down-regulation of general transcription and translation with a concurrent increase in the production of a set of specific proteins, termed heat shock proteins (HSPs) (40). These proteins serve protective roles against cellular damage caused by the stress stimuli (39). Transcription of several HSPs, such as Hsp70, have been found to be regulated by heat shock transcription factor 1 can be phosphorylated by MAPKs in vitro, the regulation of HSPs by these kinases remains undefined (59). We have demonstrated a relationship between ERK MAPK activity and cell viability. Attenuation of ERK MAPK activity with a MEK-specific inhibitor (PD98059) resulted in a loss of viability (Fig. 10), suggesting the activation of the ERK MAPK cascade may be involved in maintaining long term cell survival. This confirms previous studies demonstrating the requirement for ERK MAPK for cell survival following exposure to stress stimuli (e.g. Refs. 12, 57, and 60).

In summary, we propose a model whereby the signaling mechanism activated by heat shock for 60 min involves a deactivation of serine/threonine phosphatases that negatively regulate MEK and ERK. A recent study suggests phosphatase regulation by a chaperone heat shock protein (46). The precise mechanism of phosphatase regulation of ERK MAPK signaling will require further investigation.

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