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An Essential Role for Mitogen-Activated Protein Kinases, ERKs, in Preventing Heat-Induced Cell Death

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Abstract Stimulation of mitogen-activated protein kinases (MAPKs) or extracellular signal regulated protein kinases (ERKs) after exposure of mammalian cells to ultraviolet (UV) and X-irradiation occurs through activation of receptor tyrosine kinases via Ras/Raf/Mek/ERKs cascade. This activation of MAPKs is proposed to play a role in the replacement of damaged proteins during these stresses. Heat shock also activates MAPKs; however, the signaling cascade and the biochemical and physiological links between activation by heat and downstream effects are unknown. In this report we demonstrate that, unlike irradiation, heat induces MAPKs through ceramide metabolism to sphingosine with stimulation of Raf-1 protein kinase. The activation of MAPKs by heat does not occur in all cell types, because the step(s) downstream of ceramide to activation of Raf-1 protein kinase is missing in myeloid leukemic cells such as HL-60, U937, and K562, while it is present in NIH3T3 fibroblasts. Heat-induced MAPK activation may enhance the ability of cells to survive a severe heat shock. Blocking 60–70% of the activity of MAPK (ERK1) by stable overexpression of the dominant negative allele ERK1-KR renders NIH3T3 and K562 cells up to 100-fold more sensitive to cytotoxic effects of heat. Conversely, NIH3T3 and K562 cells stably overexpressing the wild-type ERK1 develop resistance to killing by heat. These results suggest that increased thermal sensitivity of leukemic cells to thermal stress or other cancer therapy regimens could be attributable to lack of pertinent activation of the MAPK pathway by such stresses. J. Cell. Biochem. 74:648–662, 1999.

The mitogen-activated protein kinase (MAPK) or extracellular signal regulated kinases (ERKs) signal transduction pathway is a major signaling cascade used by growth factors, neurotransmitters, hormones, and a variety of environmental stresses [Marshall, 1995]. The activation of MAPKs by growth factors, neurotransmitters, and hormones has recently been defined [Mattingly et al., 1996; van Biesen et al., 1995, 1996; Wan et al., 1996]. However, the MAPK activation pathway during stresses such as heat shock remains largely unknown.

Activation of multiple signaling pathways can lead to phosphorylation and activation of MAPK [Mattingly et al., 1996; van Biesen et al., 1995, 1996; Wan et al., 1996]. MAPKs can be activated through Ras-dependent and -independent mechanisms. In one Ras-dependent pathway, MAPK activation occurs through receptor tyrosine kinases (e.g., EGFR or PDGFR) via recruitment and activation of an exchange factor and adaptors Sos/Grb2/Shc [Pawson, 1995; Sasaoka et al., 1994]. The $G_{\beta\gamma}$ protein-coupled receptors activate Ras by the classical adaptor proteins or another recently identified exchange factor $p140^{Ras\text{-}GRF}$ [Mattingly et al., 1996; van Biesen et al., 1996]. Activated Ras-GTP then leads to phosphorylation and activation of the ser/thr protein kinase Raf-1. Raf-1 phosphorylates and activates Mek, and Mek phosphorylates MAPKs on threonine and tyrosine residues, leading to activation of the MAPKs [Crews et al., 1992; Davis, 1993; de Vries-Smith et al., 1992].

In one of the Ras-independent pathways, MAPKs can be activated through G_q protein-coupled receptors (e.g., m1 muscarinic acetylcholine receptors), platelet-activating factor receptor, and phorbol myristate acetate (PMA), leading to activation of protein kinase C (PKC)

Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated protein kinase; SAPK, stress-activated protein kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; RSK, 90-kDa ribosomal S6 kinase; PLA2, cytosolic phospholipase A2.

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[van Biesen et al., 1996]. This leads to activation of Raf/Mek/MAPKs. In lymphocytes, the G_q-protein-coupled receptors can also activate MAPKs through activation of tyrosine kinases Lyn and Syk leading to direct activation of Mek and MAPKs [Wan et al., 1996].

MAPKs have also been shown to be activated by the generation of ceramide after stimulation of cells with proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [Adam-Klages et al., 1996; Vietor et al., 1993; Yao et al., 1995]. Ceramide is produced by hydrolysis of sphingomyelin by neutral sphingomyelinases located in the plasma membrane [Wiegmann et al., 1994]. One of the known pathways of ceramide activation of MAPKs occurs through stimulation of ceramide-activated ser/thr protein kinase (CAPK), which complexes with Raf-1, causing Raf-1 activation, followed by activation of the downstream enzymes Mek/MAPKs [Yao et al., 1995]. Ceramide metabolites, sphingosine (Sph), and sphingosine-1-phosphate (SPP) can also activate MAPKs [Coroneos et al., 1996; Cuvillier et al., 1996; Wu et al., 1995]. Exogenously added SPP has been shown to activate G_i protein-coupled receptors, leading to activation of MAPKs [van Koppen et al., 1996; Wu et al., 1995].

Most mammalian cells exposed to stresses such as ultraviolet (UV) and X-irradiation, H_2O_2, and heat shock activate the MAPK as well as SAPK signaling pathways [Adler et al., 1995; Coroneos et al., 1996; Derijard et al., 1994; Engelberg et al., 1994a; Guyton et al., 1996; Kasid et al., 1996; Mivechi and Giaccia, 1995; Sachsenmaier, 1994]. Treatment of cells with UV and X-irradiation and H_2O_2 results in the activation of receptor tyrosine kinases leading to activation of Ras/Raf/Mek/MAPKs [Guyton et al., 1996; Kasid et al., 1996; Sachsenmaier, 1994]. The signaling pathway by which MAPKs are activated during heat shock has not been elucidated.

Activation of MAPKs by treatment of cells with neurotransmitters or hormones leads to expression of the genes required for cell growth and differentiation [Marshall, 1995; Seger and Krebs, 1995]. The biochemical and physiological link between MAPKs activation by different stresses is less clear. In the case of UV and X-irradiation, MAPKs activation is postulated to result in replacement of damaged proteins, rather than repair of DNA damage [Engelberg et al., 1994b; Kasid et al., 1993, 1996]. Several lines of evidence suggest that tumor cells overexpressing oncogenic Ras or Raf become more resistant to the damaging effects of irradiation. Yeast cells deficient in the activation of transcription factor GCN4 (the yeast homologue of the mammalian AP1 transcription factor) are more sensitive to damaging effects of UV irradiation [Engelberg et al., 1994b]. The role of MAPK activation in heat stress, however, has not been established. Heat stress is a potent inducer of apoptosis and necrosis among all cell types, and MAPK activation by heat may also provide protective function to various cellular components.

In this study, we investigated the signaling pathway leading to ERKs-MAPK activation after heat shock. Our results show that heat-induced MAPKs activation is not present in all cell lines. Some leukemic cells are deficient in activating MAPKs during heat shock, rendering them more susceptible to the cytotoxic effects of heat. We have also shown that MAPKs activation by heat is through the metabolism of ceramide to sphingosine with activation of Raf-1 protein kinase. The deficiency in leukemic cells that does not activate the ERK-MAPK cascade appears to reside between ceramide generation and Raf-1 protein kinase activation.

MATERIALS AND METHODS

Cell lines, Culture Conditions, and Drug Treatment

Leukemic cell lines used were K562, a human erythroleukemia cell line [Lozzio et al., 1977]; HL-60, a promyelocytic human leukemia cell line [Collins et al., 1977]; and U937, a human monoblastic leukemia cell line derived from a patient with diffuse histiocytic lymphoma [Sundstrom and Nilsson, 1976]. All leukemic cell lines were maintained in Dulbeccos' minimal essential medium (DMEM) supplemented with 15% fetal calf serum (FCS). NIH3T3 cells, a murine fibroblast cell line, was maintained in DMEM supplemented with 10% FCS. All experiments, except when otherwise indicated, were performed with cells that had been preincubated for 48 h in DMEM supplemented with 0.5% FCS to reduce background activity of ERKs -MAPK.

TNF-α, epidermal growth factor (EGF), and all sphingolipid metabolites were purchased from Sigma Chemical Co. or Biomol. C2-Ceramide, (1S, 2R)-D-erythro-2-(N-myristolamino)-1-phenyl-1-propanol (D-MAPP), and sphingo-

sine were dissolved in ethanol. Sphingosine was complexed with bovine serum albumin (BSA) (0.2%) and subsequently incubated with the cultured cells [Davis, 1988]. Control cells were treated with the solvents alone.

Transfections and Heat Survival Assays

Stable transfections. NIH3T3 cells used in stable transfection experiments contained constitutively active Ha-Ras and were neomycin resistant (a gift from Dr. A. Giaccia, Stanford University) [Koong et al., 1994; Mivechi and Giaccia, 1995]. Cells were cotransfected using calcium phosphate [Sambrook et al., 1989] with expression constructs encoding wild-type ERK1 (HA-ERK1) or its dominant negative allele HA-ERK1-KR and with vectors containing the hygromycin gene as a selectable marker. Cells were then incubated at 37°C for 48 h and harvested with trypsin. Cells were serially diluted in DMEM supplemented with 10% FCS and 400 μg/ml of hygromycin (Sigma). Individual colonies were selected after 8 days' incubation at 37°C and tested for expression of transfected genes using antibody specific for hemaglutinin (HA) (12CA5). K562 cells were stably transfected with the same plasmid cDNAs, except that electroporation was used.

Transient transfections. For transient transfection assays, cells were transfected by electroporation (BioRad, Gene Pulser) with 0.2 μg of Renila luciferase, 5 μg of Gal4-ElK1, and 5 μg (Gal4)5-luciferase, with carrier DNA to a total of 15 μg DNA. At 48 h after transfection, cells remained untreated or were treated with 50 nM of PMA for 20 min, rinsed with phosphate-buffered saline (PBS), and incubated in complete medium at 37°C for 4 h. Cells were then lysed and firefly luciferase and Renila luciferase (as a control for transfection frequency) were determined from 20 μg of protein by the Dual Luciferase System (Promega, Madison, WI).

Cellular survival assays were performed by colony formation analysis as described previously [Mivechi et al., 1994b]. The surviving fraction was determined as the (number of colonies obtained after treatment)/(number of cells plated) divided by (number of colonies obtained without pretreatment)/(number of cells plated).

The surviving fraction of heat-treated K562 cells were obtained as above, except that cells were grown in DMEM supplemented with 15% FCS containing 1% methylcellulose as the supporting medium [Mivechi, 1988].

Immunoblotting and Immune Complex Kinase Assays

After various treatments as indicated in the figure legends, cells were lysed in SDS sample buffer [Laemmli, 1970], and an aliquot from each sample was precipitated with 10% TCA. Protein concentration was determined by bicinchoninic acid (Pierce). A total of 30 μg of protein from each sample was analyzed by immunoblotting [Mivechi and Giaccia, 1995], using the antibodies indicated under Results, and ECL (Amersham) was used as the detection system.

For immune complex kinase assays, cells were lysed in lysis buffer (50 mM sodium β-glycerophosphate, pH 7.2, 10 mM magnesium chloride, 5 mM ethyleneglycol tetra-acetic acid, 1 mM EDTA, 10 mM potassium phosphate monobasic (KH_2PO_4), 1 mM sodium orthovanadate, 0.2 mM PMSF). The protein concentration of the lysate was determined by bicinchoninic acid and 300 μg of protein was incubated by rotation with 5 μg of the appropriate antibody for 1 h at 4°C. 25 μl of a 50% solution of protein A was added to each sample and the mixture rotated for an additional 1 h at 4°C. The immunoprecipitates were washed with lysis buffer 3 times and used in a kinase reaction using appropriate purified substrates [Kim et al., 1997]. Immunoprecipitates were incubated for 20 min at 37°C in 10 μl of kinase buffer (20 mM β-glycerophosphate pH 7.3, 5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and 0.2 mM PMSF) containing 25 μM of unlabeled ATP, 10 μCi of ^{32}P-γ-ATP and 1 μg of the substrate. The substrates, GST-Jun (1–79) and 6His-Mek-k97m (kinase inactive) were purified using glutathione-agarose beads (Sigma) or Ni-Column (Pharmacia), respectively, following the manufacturers' instructions. Myelin basic protein (MBP) was purchased from UBI. The reactions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gels were exposed to X-ray film and quantitated by Phosphorimager (Molecular Dynamics). The data are presented as fold increase after treatment by dividing the amount of ^{32}P counts present in treated groups by the counts in the control group.

The anti-ERK antibodies used for immunoblotting include anti-phosphospecific MAPK antibodies (New England Biolabs) that recognize

the phosphorylated form of ERKs and anti-MAPK antibody k23 (Santa Cruz), which recognizes the ERK proteins. The anti-MAPK (c16) (anti-ERKs) and c14 (anti-ERK2) (Santa Cruz Biotechnology) were used for immunoprecipitation. Anti-Raf-1 (c12) and JNK1 (c17) polyclonal antibodies recognizes c-Raf-1(p74 kDa) and JNK1 (p54 kDa) (Santa Cruz). All antibodies used were cross-reactive to both human and mouse protein kinases.

RESULTS

Leukemic Cells Lack Heat-Induced MAPK Activity

Exposure of mammalian cells to elevated temperatures (43–45°C) induces cell death [Li and Mivechi, 1986]. Among the tissues tested, the normal hematopoietic progenitors and some leukemic cells are most heat sensitive [Li and Mivechi, 1986; Mivechi, 1988; Mivechi et al., 1992, 1994a]. In the experiment shown in Figure 1, exposure of HL-60 or U937 leukemic cells to a heat dose of 43°C for 60 min reduced cell survival to 3×10^{-3} and 4×10^{-2}, respectively. K562 cells that contain constitutively active MAPKs due to a bcr/abl translocation were relatively heat resistant. Exposure to 43°C for 60 min reduced cell survival to only 4×10^{-1}. NIH3T3 cells and other fibroblast-derived cell lines gave similar results to K562 cells [Mivechi et al., 1994a].

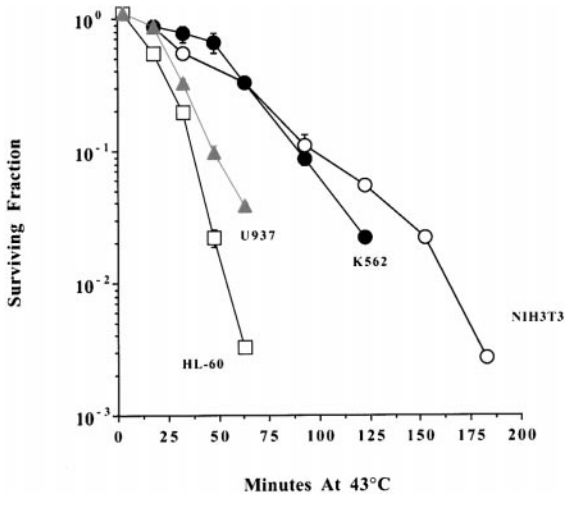

Fig. 1. Response of leukemic and nonleukemic cells to heat shock. HL-60 (□), U937 (▲), K562 (●), and NIH3T3 (○) cells were heated at 43°C for increasing lengths of time. Cell survival was estimated by colony formation assays [Mivechi et al., 1994a].

To test the hypothesis that leukemic cells are deficient in activation of the potentially protective ERKs-MAPK after heat shock, we measured the ability of leukemic and nonleukemic cells to activate MAPKs by heat. Cells were heated to 43°C for 20 min, and the activation of MAPKs was measured by immunoblot analysis using antibody specific for the phosphorylated (active) forms of ERKs. Figure 2A shows that ERK2-MAPK was not activated in leukemic K562, HL-60, and U937 cells, whereas it was strongly activated in heated NIH3T3 fibroblasts relative to control cells. Other nonleukemic human and murine-derived cell lines, such as HeLa, A549, Ht1080, and Rat-1, were also shown to activate MAPKs by heat shock (data not shown). Pretreatment of cells with PMA increased ERK2-MAPK activity in all cell lines. These results suggest that leukemic cells are capable of activating MAPKs by agents other than heat but not by heat. MAPKs also failed to be activated in the leukemic cell lines when the temperature or length of heating times were increased (data not shown). The relative abundance of nonphosphorylated MAPKs (ERK1 and ERK2) are shown in the middle panel. In the cell lines tested here, we found ERK2-MAPK to be the more abundant species (Fig. 2A, middle).

Activity of MAPKs after treatment of cells with heat (43°C) or PMA was also determined using immune complex kinase assays (Fig. 2B). Activated phosphorylated ERKs were immunoprecipitated from treated cells, as described under Methods and Materials, and tested for their ability to phosphorylate MBP. Phosphorylation of MBP from heat-shocked cells was detected only when ERKs were immunoprecipitated from NIH3T3 cells, and not from K562, HL-60, or U937 cells. By contrast, MBP phosphorylation was detected for ERKs immunoprecipitated from all cell lines after PMA treatment. These results are similar to the results obtained with the immunoblot analysis shown in Figure 2A and confirm the hypothesis that leukemic cells cannot activate ERK-MAPKs in response to heat shock.

The stress- and heat-inducible MAPK family member, SAPK/JNK, has been shown to be involved in stress-induced growth arrest, differentiation, and apoptosis [Adler et al., 1995; Verheij et al., 1996; Xia et al., 1995]. This signaling cascade includes activation of Rac/MEKK1/JNKK/JNK, which leads to the phosphorylation and activation of c-Jun transcription factor

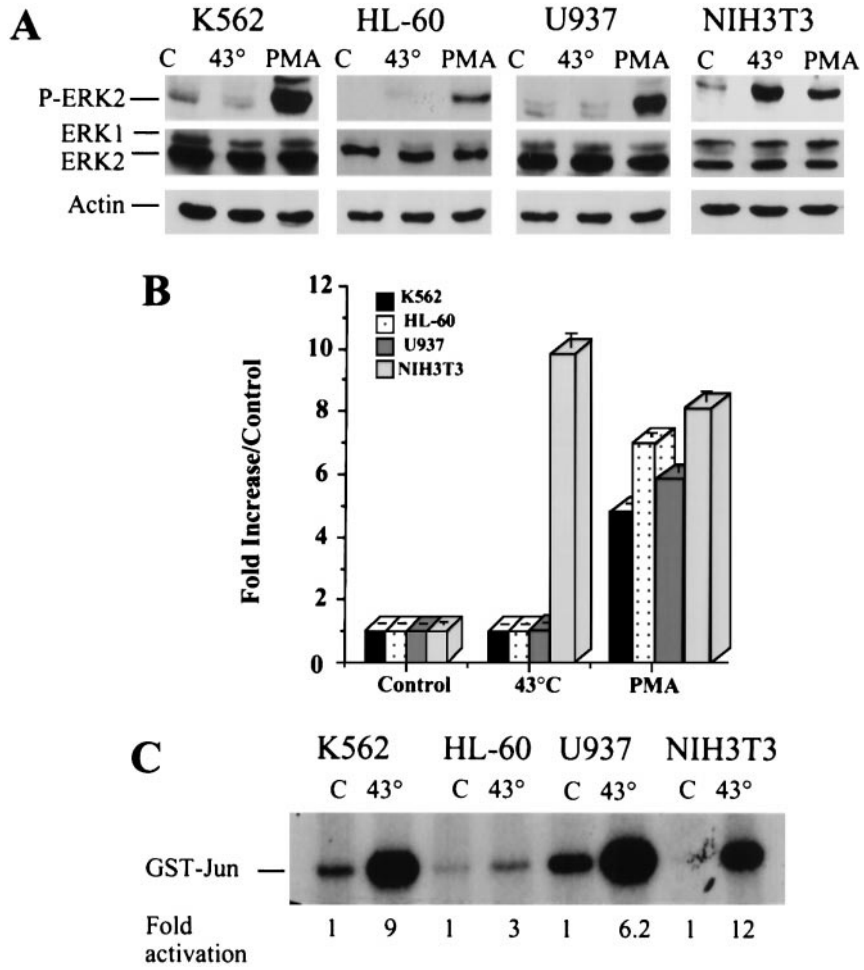

Fig. 2. MAPK/ERKs are not activated in leukemic cells during heat shock. **A:** Immunoblot analysis. Cells were untreated (C), heated at 43°C for 20 min (43°C), or pretreated with 50 nM PMA for 20 min (PMA). Top: Activity of MAPKs was determined by immunoblotting using equal amounts of protein from each sample and antibody specific for the phosphorylated forms ERK2-MAPK. Middle: The same blot was probed with antibody that recognizes unphosphorylated ERK1 (44 kDa) and ERK2 (42 kDa) proteins. Lower: The same blot was probed with antibody to actin (45 kDa). **B:** Immune complex kinase assays. Cells were treated as in A, and the activity of MAPKs measured by immunoprecipitation of ERKs, followed by in vitro phosphorylation using MBP as substrate. The ^{32}P-labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by quantitation with Phosphorimager. **C:** Immune complex kinase assays of JNK. Cells were untreated (C) or heated at 43°C for 20 min (43°C). Activity of JNK kinase was determined by immunoprecipitation of JNK followed by in vitro phosphorylation of GST-Jun (1–79) (approximately 33 kDa). The ^{32}P-labeled proteins were analyzed by by SDS-PAGE, followed by exposure to X-ray film and quantitation with Phosphorimager.

[Derijard et al., 1994; Minden et al., 1995]. To investigate whether leukemic cells were capable of activating the JNK signaling cascade during heat shock, JNK activity was measured by immune complex kinase assays (Fig. 2C). JNK1 was immunoprecipitated from control or heated (43°C) cell lysates using antibody specific for JNK1. Activated JNK was tested for its ability to phosphorylate the N-terminal region of c-Jun, GST-Jun (1–79). All cell lines showed increases in JNK activity after heat shock, suggesting that leukemic cells are specifically deficient for activation of the ERK-MAPK pathway, but not for the SAPK/JNK pathway.

Heat-Induced MAPK Activation Occurs Through Ceramide Metabolism With Activation of Raf-1, a Step That Is Deficient in Leukemic Cells

We then sought to determine the cause of the deficiency of leukemic cells to activate ERK-MAPKs by heat shock. To achieve this, we first needed to identify the heat-induced signaling pathway leading to MAPK activation. Treatment of cells with heat shock has been postu-

lated to affect primarily the lipid bilayer of the plasma membrane. Consistent with this view, changes in lipid second messengers, such as ceramide or arachidonic acid, are induced by heat shock [Chang et al., 1995; Jurivich et al., 1996; Verheij et al., 1996]. Since the sphingolipids are also potent inducers of several MAPK family members after a variety of treatments, we explored the possibility that ceramide may be involved in MAPK activation by heat shock. Cells were treated with the membrane-permeable N-acetyl sphingosine, C2-ceramide, and MAPK activation was determined by immunoblot analysis using antibody specific for the phosphorylated (active) forms of ERK2-MAPKs (Fig. 3). Leukemic cells treated with C2-ceramide were deficient in activating MAPK but were able to activate ERK2-MAPK when treated with PMA. Immune complex kinase assays of immunoprecipitated ERKs from control, C2-ceramide, or PMA-treated leukemic and NIH3T3 cells, followed by in vitro phosphorylation of MBP (Fig. 3B) also showed that the ERK-MAPKs were not activated in leukemic cells by C2-ceramide but were activated by PMA. By contrast, ERK-MAPKs from NIH3T3 cells were activated by either C2-ceramide or PMA. These results suggest that heat-induced

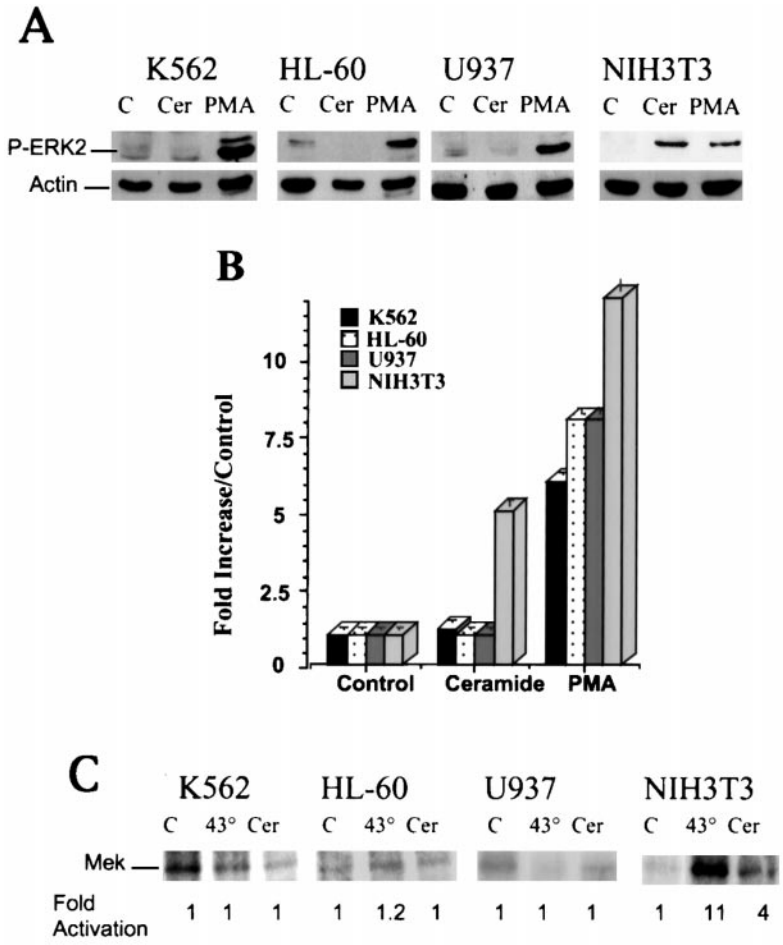

Fig. 3. C2-Ceramide does not activate MAPK cascade in leukemic cells. **A:** Immunoblot analysis. Cells were untreated (C), or treated with 50 μM C2-ceramide (Cer) for 20 min at 37°C. Top: Activity of MAPKs was determined by immunoblotting using antibody specific for the phosphorylated forms of ERK2-MAPKs. Bottom: The same blot was probed with antibody to actin (45 kDa). **B:** Immune complex kinase assays. Cells were treated as in A, and the activity of MAPKs was measured by immunoprecipitation of ERKs followed by in vitro phosphorylation using MBP as substrate. The ^{32}P-labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by quantitation with Phosphorimager. **C:** Immune complex kinase assays of Raf-1. Cells were untreated (C), heated at 43°C for 20 min (43°C), or treated with 50 μM C2-ceramide (Cer) for 20 min at 37°C. The activity of Raf-1 kinase was determined by immunoprecipitation of Raf-1, followed by phosphorylation of kinase inactive Mek as substrate. The ^{32}P-labeled proteins were analyzed by SDS-PAGE, followed by exposure to X-ray film and quantitation with Phosphorimager.

MAPK activation may be through the generation of ceramide metabolism.

Ceramide can be produced by sphingomyelin breakdown at different subcellular sites [Wiegmann et al., 1994]. Acidic sphingomyelinases hydrolyze sphingomyelin to ceramide in endosomes; this activity has been linked to NF-κB activation. Neutral sphingomyelinases hydrolyze sphingomyelin to ceramide in the membrane bilayer; this activity has been linked to the MAPK signaling pathway [Cuvillier et al., 1996; Verheij et al., 1996; Wu et al., 1995]. Although the details of the latter pathway are not entirely clear, ceramide has been shown to activate ceramide-activated protein kinase (CAPK), which leads to direct activation of the cytoplasmic serine/threonine protein kinase Raf-1 [Yao et al., 1995]. CAPK has not yet been clearly characterized. We therefore investigated whether the deficiency of leukemic cells to activate MAPKs in the presence of C2-ceramide occurs at the step of ceramide activation of Raf-1 (Fig. 3C). Raf-1 was immunoprecipitated from control, heated, or C2-ceramide-treated cells, using an antibody specific for Raf-1 and used to phosphorylate kinase-inactive Mek1 in an immune complex kinase assay. Mek1 failed to be activated by Raf1 from heated (43°C) or C2-ceramide-treated leukemic cells but was activated by Raf1 from both heated and C2-ceramide-treated NIH3T3 cells. These results suggest that the deficiency to activate MAPKs in heated leukemic cells occurs via the Raf activation pathway.

The stimulation of TNF-α receptor has been shown to lead to generation of ceramide and activation of CAPK/Raf-1/MAPKs [Yao et al., 1995]. However, it is unlikely that the heat-induced MAPK activation by ceramide occurs via TNF-α because NIH3T3 cells lack the 55-kDa TNF receptor 1. Treatment of NIH3T3 cells with TNF-α (20 ng/ml for 20 min) did not stimulate MAPKs in immunoblotting and immune complex kinase assays (data not shown). By contrast, MAPKs were activated in TNF-α-treated leukemic cells that possess this TNF receptor [Yao et al., 1995].

The ceramide metabolites sphingosine and sphingosine-1-phosphate have recently been shown to be potent activators of MAPKs [Coroneos et al., 1996; Cuvillier et al., 1996; Wu et al., 1995]. Sphingosine presumably acts through sphingosine-1-phosphate [Olivera and Spiegel, 1993]; however, exogenously added sphingosine-1-phosphate does not act as a second messenger but, rather, activates MAPKs through G_i protein-coupled receptors [Wu et al., 1995]. We therefore investigated whether sphingosine can activate MAPKs in leukemic cells. HL-60, U937, K562, and NIH3T3 cells were treated with sphingosine and MAPK activation measured by immunoblotting using antibody specific for the phosphorylated form of ERK2-MAPKs (Fig. 4A). The results observed for sphingosine treatment were similar to those seen with heat stress. ERK2 was activated in sphingosine-treated NIH3T3 cells only, and not in leukemic cells. These observations suggest that ceramide generation induced with heat stress could result in

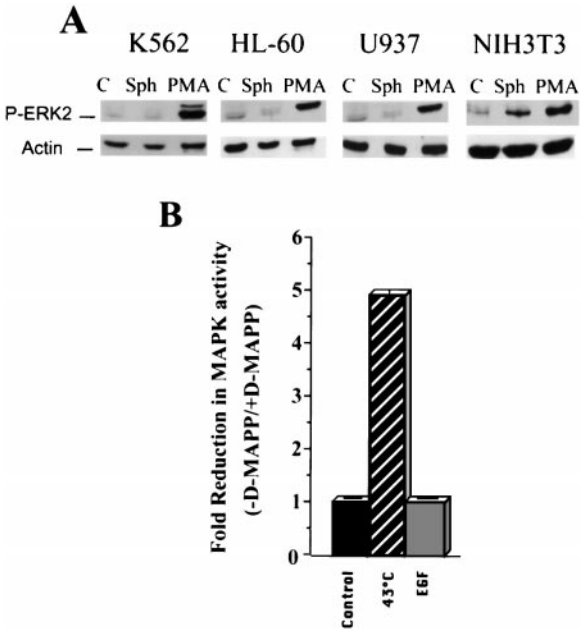

Fig. 4. Heat-induced MAPK activation is mediated by generation of sphingosine. **A:** Immunoblot analysis. Cells were treated with 10 μM sphingosine (Sph) for 20 min at 37°C or with 50 nM of PMA for 20 min or left untreated as control (C). Top: Activity of MAPKs was determined by immunoblotting equal amounts of protein from each sample using antibody specific for the phosphorylated forms of ERK2-MAPK. Bottom: The same blot was probed with antibody to actin (45 kDa). **B:** Immune complex kinase assays. NIH3T3 cells were untreated (control) or pretreated with 20 μM of D-MAPP for 24 h. Pretreated (+D-MAPP) and control cells (−D-MAPP) were not subjected to heat (control) or heated at 43°C (43°C) for 20 min, or treated with 50 ng/ml of EGF for 20 min (EGF). Activity of MAPKs was measured by immunoprecipitation of ERKs followed by in vitro phosphorylation using MBP as substrate. The ^{32}P-labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by quantitation with Phosphorimager. The data is reported as fold reduction in MAPK activity in groups that were not treated with D-MAPP divided by the same group that were pretreated with D-MAPP.

the activation of Raf-1 via a ceramidase and sphingosine kinase-dependent pathway [Coroneos et al., 1996; Cuvillier et al., 1996; Wu et al., 1995].

Recently, it was shown that alkaline ceramidase can be inhibited specifically by D-MAPP ((1S, 2R)- D-erythro-2-(N-myristolamino)-1-phenyl-1-propanol) both in vitro and in vivo [Bielawska et al., 1996]. To test directly whether sphingosine production from ceramide is responsible for heat-induced MAPK activation, we inhibited alkaline ceramidase in NIH3T3 cells by treating cells with D-MAPP before heating. Cells pretreated with D-MAPP showed 5-fold reduction in heat-induced ERK-MAPK activation compared to cells not pretreated with the ceramidase inhibitor in immune complex kinase assays (Fig. 4B). To show that the Ras/Raf/MAPK is not altered by treatment with D-MAPP, the following control experiment was performed. Cells untreated or pretreated with D-MAPP, were then treated with EGF, a known Ras activator. No differences in MAPK activation via a Ras/Raf pathway activity were detected. Taken together, these results suggest that heat-induced MAPK activation is mediated mainly by ceramide metabolism to sphingosine.

Another signaling cascade that results in MAPK activation involves receptor tyrosine kinases or heterotrimeric G proteins leading to activation of Ras-GTP [Crespo et al., 1994; de Vries-Smith et al., 1992; Hawes et al., 1995; van Biesen et al., 1995]. To rule out the possibility that the heat-induced activation of MAPKs occurs via a Ras-dependent pathway, we used NIH3T3 cells that stably overexpress the dominant negative mutant of Ras (N17) [Koong et al., 1994]. In this experiment, the anti-ERK antibody used (k23, Santa Cruz) recognizes both phosphorylated and nonphosphorylated forms of ERK1 and ERK2. The phosphorylated (activated) forms of ERKs are identified by their slower mobility by SDS-PAGE. Immunoblot analysis using antibody specific for ERK1 and ERK2 suggested that heat-shocked NIH3T3-Ras (N17) cells resulted in activation of MAPKs to the same extent as control cells overexpressing wild-type Ras (Fig. 5A). Immune complex kinase assays using MBP as a substrate showed similar results (data not shown). To demonstrate that the Ras/Raf/MAPK pathway was functional in the NIH3T3 fibroblasts, the following control experiment was carried out, as shown in Figure 5B. NIH3T3 cells that overexpress wild-type Ras or that express the N17 dominant negative mutant of Ras were treated with EGF, to activate the Ras/Raf/ MAPK cascade. The Ras N17 cells showed a greater than 70% reduction in activation of ERK1 and ERK2 in response to EGF.

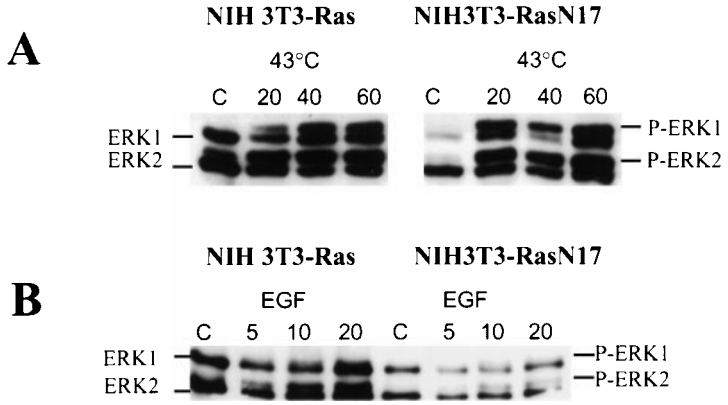

Fig. 5. Heat-induced MAPKs activation is not dependent on Ras. **A:** Immunoblot analysis. NIH3T3-Ras cells (containing IPTG-inducible Ras) and NIH3T3-RasN17 cells, which constitutively express the RasN17 dominant negative mutant, were treated with 20 mM IPTG for 24 h at 37°C to induce Ras expression [Mivechi and Giaccia, 1995]. Cells were then held as controls (C) or heated at 43°C for 20, 40, or 60 min. Activity of MAPKs was determined by immunoblotting using antibody (k23) specific for ERK1 and ERK2. The activation of ERK1 and ERK2 is denoted by retardation of the active, phosphorylated P-ERK1 and P-ERK2 forms of the enzymes in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as indicated. **B:** Immunoblot analysis. NIH3T3-Ras and NIH3T3-RasN17 were treated with 20 mM IPTG for 24 h at 37°C to induce Ras expression. Cells were then held as controls (C) or were untreated (C) or treated with 50 ng/ml of EGF for 5, 10, and 20 min at 37°C. Activity of MAPKs was determined by immunoblotting using antibody specific for ERK1 and ERK2-MAPKs as in A.

The above results show that heat-induced activation of MAPKs occurs in fibroblasts in the absence of an active form of the Ras gene, suggesting that the heat-induced activation of MAPKs occurs via a Ras-independent pathway. To further document that this is the case, we looked at an activation event located upstream of Ras in the receptor tyrosine kinases signaling pathway [Li et al., 1993], that is, the activation of Shc and its association with Grb2. Using the immunoblotting assay, no increase in tyrosine phosphorylation of Shc was detected after heat shock in NIH3T3 or A431 cells, which are more responsive to activation of Ras via Shc/Grb2 because they are known to contain a greater number of EGF receptors (data not shown). Likewise, no increase in the association and co-immunoprecipitation of Shc with Grb2 was detected when immunoprecipitated Shc was immunoblotted using Shc- or Grb2-specific antibodies (data not shown). However, Shc was activated when cells were treated with 50 ng/ml EGF for 10 min. Taken together, these results suggest that the primary pathway of heat-induced MAPK activation is not via a Ras-dependent mechanism and does not involve activation of receptor tyrosine kinases.

Activation of PKC through treatment of cells with PMA or G protein-coupled receptors or arachidonic acid has also been shown to stimulate MAPKs activity [Berra et al., 1995; Hawes et al., 1995; Hii et al., 1995; van Biesen et al., 1996]. Activation of PKC leads directly to activation of Raf-1 or Mek protein kinases, leading to an increase in MAPK activity [Hawes et al., 1995; Kolch et al., 1993; Marquardt et al., 1994; Siddhanti et al., 1995]. To rule out the possibility that the activation of PKC by heat may lead to MAPK activation, we treated different leukemic (HL-60, K562, U937) and nonleukemic cells (NIH3T3) with PMA. MAPK activation was measured by immunoblotting as shown in Figures 2, 3, and 4. The results showed that MAPK was activated in all cell lines tested, indicating that activation of PKC, which can lead to activation of MAPKs, was intact in leukemic cells. The results also suggested that PKC was not the primary signaling molecule for heat-induced MAPK activation.

PKC is involved in many signaling events. It has recently been shown to activate sphingosine kinase and is also known to be the target for sphingosine [Cuvillier et al., 1996; Hannun and Bell, 1989; Senisterra and Epand, 1992]. The activated MAPKs are also able to stimulate PLA_2, which could result in generation of arachidonic acid and further activation of MAPKs [Hii et al., 1995; Lin et al., 1993; Warner et al., 1993]. To show more precisely whether heat-induced MAPKs activation is modulated by PKC activation, NIH3T3 cells were depleted of intracellular PKC by treatment of cells with 1 μM PMA for 24 h to block signaling through PKC α, δ, and ϵ [Hawes et al., 1995; Hii et al., 1995; Marquardt et al., 1994]. This treatment did not cause any cytotoxicity to NIH3T3 cells, but the depleted cells were deficient in ERK-MAPK activation when re-exposed to PMA as previously reported (Fig. 6, lane 3). However, depleted cells remained capable of activating MAPKs by heat shock (lane 5), suggesting that heat-induced MAPKs activation is independent of PKC.

Activation of MAPKs by Heat Has a Protective Effect on Heat-Induced Cell Death

The activation of MAPKs by different environmental stresses has been suggested to be involved in the repair of proteins, possibly from damage that might occur during exposure to such stresses [Engelberg et al., 1994b; Kasid et al., 1993, 1996]. Fibroblast cells blocked in their ability to activate MAPK have been shown to be less effective in surviving exposure to H_2O_2 than normal cells [Guyton et al., 1996]. It is conceivable that the lack of activation of MAPKs in leukemic cells by environmental stresses may be the reason for their increased sensitivity to various cytotoxic treatments. To investigate the contribution of the activation of MAPKs to the ability of cells to survive a heat shock, we established several lines of NIH3T3 and K562 cells

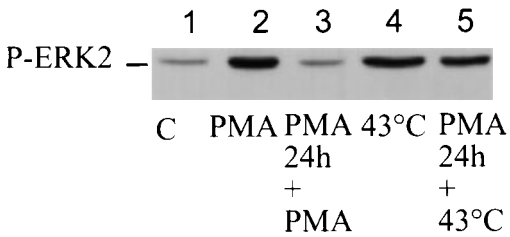

Fig. 6. Heat-induced MAPKs activation is independent of phosphokinase C (PKC). NIH3T3 cells were untreated (**lane 1**), treated with 1 μM PMA for 5 min (**lane 2**), treated with 1 μM PMA for 24 h and rechallenged with 1 μM PMA for 5 min (**lane 3**), heated at 43°C for 20 min (**lane 4**), or treated with 1 μM PMA and heated at 43°C for 20 min (**lane 5**). Activity of MAPKs was determined by immunoblotting using antibody specific for the phosphorylated forms of MAPKs.

that stably overexpress hemaglutinin-tagged-wild-type (HA-ERK1) or the dominant negative allele of ERK1 (HA-ERK1-KR) as described under Materials and Methods. The level of ERK1 and ERK1-KR expressed in these lines was monitored by immunoblotting, using antibody to hemagglutinin (Fig. 7A,B).

ERK1 activation has been shown to increase ELK1 transcription factor phosphorylation and increase its transcriptional activity directly [Derijard et al., 1995]. To test the ability of ERK1 or ERK1-KR to activate or inhibit the activity of downstream transcription factor ELK1, K562 and NIH3T3 cells stably overexpressing these genes were transiently transfected with Gal4-ELK1 and (Gal4)5-luciferase reporter plasmids [Derijard et al., 1995]. Two days after transfection, cells were treated with PMA to activate MAPKs, and ELK1 transcription factor and luciferase activity were measured. The results show that overexpressed ERK1 upregulated the expression of luciferase and ERK1-KRs downregulated the activity of ELK1 transcription factor in both NIH3T3 and K562 cells (Fig. 7C). ERK1-KR-expressing cells showed a 60–70% reduction in luciferase activity, suggesting that effective inhibition of the MAPK pathway was achieved in these cells.

To test the possibility that heat-induced ERK-MAPK activation is protective against cytotoxic effects of heat, K562 and NIH3T3 cells stably overexpressing ERK1 or ERK1-KR were heated, and the surviving fractions of different lines were assessed by colony formation analysis. The results showed that blocking ERK1 kinase activity resulted in an up to 100-fold increase in sensitivity to heat shock in cells stably overexpressing ERK1-KR when compared with the parent cell line or cells transfected with hygromycin as a selectable marker only (Fig. 7D). Conversely, the stable overexpression of ERK1 protein kinase resulted in resistance to killing by heat shock. NIH3T3 and K562 cells overexpressing wild-type ERK1 were 2- to 3-fold, and 5- to 10 fold, respectively, more resistant to heat (Fig. 7D). These results strongly suggest that ERK1 activation by heat can provide protection against heat-induced cell death.

DISCUSSION

In this report we show that heat stress induces MAPKs via a Ras-independent signaling pathway. This is in marked contrast with other stresses, such as H_2O_2 and UV- or X-irradiation, which involve the activation of receptor tyrosine kinases leading to Ras activation [Engelberg et al., 1994a; Guyton et al., 1996; Kasid et al., 1996; Whitmarsh et al., 1995]. Our data suggest that ceramide metabolism is the mediator of a Raf/Mek/MAPK activation cascade during heat stress. Whether stresses other than heat also use similar mechanism to activate the MAPK pathway is unknown. It has been known, however, that blocking Ras inhibits H_2O_2-induced MAPK activation, suggesting that, at least in the case of H_2O_2, Ras/Raf/Mek is the major inducer of MAPKs [Guyton et al., 1996]. In one study, ceramide generated after treatment of cells with proinflammatory cytokines such as TNFα has been linked to MAPK activation [Yao et al. 1995, 1995]. However, heat and TNFα-induced ceramide metabolism do not seem to share a common pathway or intracellular location, since NIH3T3 cells lacking TNF-receptor 1 activate MAPKs during heat stress, while leukemic cells activate the MAPKs pathway by TNFα [Yao et al., 1995], but not by heat. In contrast to TNFα-signaling, where ceramide possibly activates CAPK, which directly phosphorylates Raf-1, our data suggest that ceramide generated during heat stress is metabolized to sphingosine and sphingosine-1-phosphate leading to activation of Raf-1 and MAPKs. Blocking the generation of sphingosine from ceramide by pretreatment of cells with D-MAPP inhibited heat-induced MAPK activation. The view that ceramide metabolism and not ceramide itself is necessary for heat-induced MAPKs activation is further supported by the fact that D-MAPP treatment which results in the accumulation of ceramide in the cells by three- to eightfold [Bielawska et al., 1996] does not lead to an increase in MAPK activation (Fig. 4B).

The involvement of G-proteins in heat-induced MAPKs activation cannot be directly excluded by our data. However, it has been shown that G_i-coupled receptors use a Ras-dependent pathway to activate MAPKs and G_q- and G_0-coupled receptor signaling to MAPKs are PKC dependent [Hawes et al., 1995; van Biesen et al., 1995, 1996]. Our results demonstrate that neither Ras nor PKC is primarily responsible for activation of MAPKs by heat (Figs. 5, 6), so it seems unlikely that G-proteins are involved in heat-induced MAPKs activation.

As is the case with other stresses, heat shock activates several signaling pathways, including

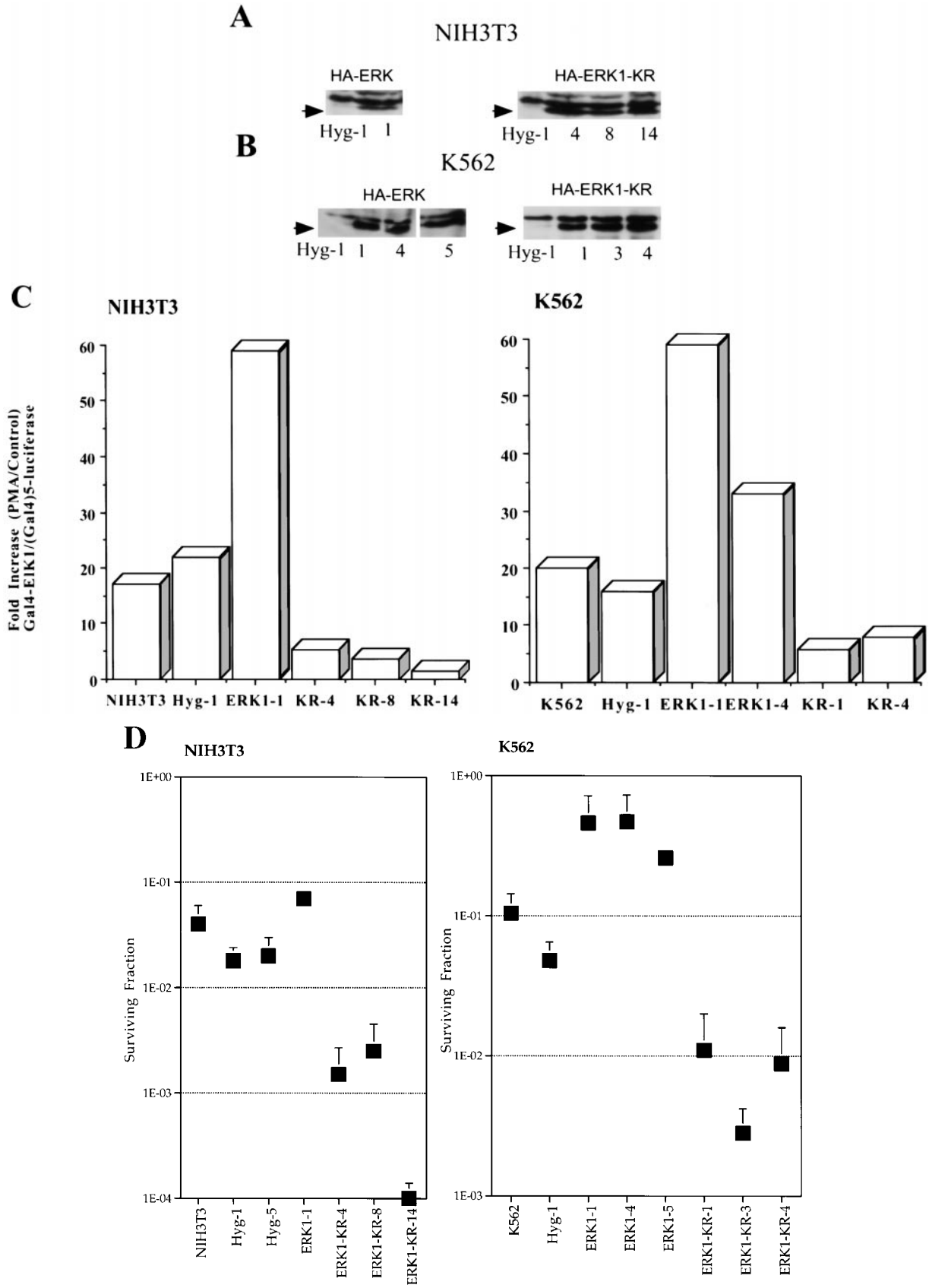
A
NIH3T3
HA-ERK
Hyg-1 1
HA-ERK1-KR
Hyg-1 4 8 14
B
K562
HA-ERK
Hyg-1 1 4 5
HA-ERK1-KR
Hyg-1 1 3 4
C
NIH3T3
K562
Fold Increase (PMA/Control)
Gal4-Elk1/(Gal4)5-luciferase
0 10 20 30 40 50 60
NIH3T3 Hyg-1 ERK1-1 KR-4 KR-8 KR-14
K562 Hyg-1 ERK1-1 ERK1-4 KR-1 KR-4
D
NIH3T3
K562
Surviving Fraction
1E+00 1E-01 1E-02 1E-03 1E-04
NIH3T3 Hyg-1 Hyg-5 ERK1-1 ERK1-KR-4 ERK1-KR-8 ERK1-KR-14
K562 Hyg-1 ERK1-1 ERK1-4 ERK1-5 ERK1-KR-1 ERK1-KR-3 ERK1-KR-4

Figure 7.

MAPK, JNK, and p38 protein kinases [Adler et al., 1995; Bensuade et al., 1993; Mivechi and Giaccia, 1995]. Activation of these proline-directed protein kinases results in activation of the transcription factors ELK1, c-Jun (AP1), and ATF2 [Derijard et al., 1994, 1995; Gupta et al., 1995]. Heat shock also activates the as yet unidentified signaling pathway that leads to thermotolerance, defined as a transient resistance to a subsequent heat challenge. Thermotolerance has been firmly correlated with the expression of the heat shock proteins (HSPs) [Landry et al., 1989; Li et al., 1991]. The control of HSP expression is through the activation of heat shock transcription factor-1 (HSF-1) [Abravaya et al., 1991; Mivechi et al., 1992; Zimarino and Wu, 1987], which has recently been shown to be phosphorylated by one or more of the proline directed protein kinases [Kim et al., 1997; Knauf et al., 1996; Mivech and Giaccia, 1995]. Heat stress is also an excellent inducer of programmed cell death, which in the case of heat shock may involve the activation of the JNK signaling cascade [Verheij et al., 1996; Xia et al., 1995]. It is noteworthy that cells overexpressing HSPs, i.e., when they are in the "thermotolerant state," are protected from various environmental imposed damages, including apoptosis. As suggested in other systems, there may be a fine balance between the activity of these signaling pathways after heat shock and the final cellular response, depending on how strongly these various pathways are activated.

As our results show, leukemic cells are poor inducers of MAPKs by heat shock, possibly due to a deficiency located downstream of sphingosine or sphingosine-1-phosphate that has recently been linked to cell survival signals [Cuvillier et al., 1996]. The signaling block downstream of sphingosine in leukemic cells could be the explanation for diverging reports on the biological effects of the sphingolipids. Sphingosine was recently shown to induce apoptosis in leukemic HL60 and U937 cells, while it functions as second messenger for proliferation and cell survival in fibroblasts and mesangial cells [Coroneos et al., 1995; Jarvis et al., 1996; Olivera and Spiegel, 1993; Zhang et al., 1990]. It is not surprising that bone marrow progenitors and leukemic cells are also very poor inducers of HSF-1 activation and HSP synthesis [Mivechi et al., 1991]. Leukemic cells and normal bone marrow progenitors also have a dramatically reduced thermotolerance response [Mivechi, 1988; Mivechi et al., 1992, 1994a]. As we have shown, various leukemic cells do not activate MAPKs by heat; however, they can efficiently activate the JNKs during heat shock and other stresses. JNKs activation has been linked to the induction of apoptosis [Ichijo et al., 1997; Johnson et al., 1996; Verheij et al., 1996], which may explain why leukemic cells are sensitive to various stresses. This conclusion is supported by the data shown in Figure 7, where stable overexpression of ERK1 enhances the ability of cells to survive a severe heat shock, whereas cells overexpressing the dominant negative allele ERK1-KR are heat sensitive. These data clearly suggest an important role for the activation of the MAPK-ERK pathway in intrinsic cellular heat sensitivity, i.e., the inherent level of heat sensitivity, in contrast to thermotolerance, the inducible, transient state of heat resistance.

Figure 8 summarizes the pleiotropic effects of heat that may lead to the activation of various signaling pathways via production of ceramide. The heat-induced generation and metabolism of ceramide could lead to activation of proline directed protein kinases, which may then mediate the downstream effects observed during heat stress. Various proline-directed protein kinases are induced by heat and may regulate stress response (HSP synthesis, thermotolerance) and apoptosis or may facilitate the repair

Fig. 7. Stable overexpression of ERK1 and ERK1-KR in NIH3T3 and K562 cells alters their thermal sensitivity. **A,B:** Immunoblot analysis of cell lysates stably overexpressing HA-ERK1 and HA-ERK1-KR using antibody specific to hemagglutinin (HA). The expression of HA-ERK1 and HA-ERK1-KR in clones HA-ERK1–1 and HA-ERK1-KR-4, -8, and -14 for NIH3T3 cells and HA-ERK1–1, -4, and -5 and HA-ERK1-KR-1, -3, and -4 for K562 cells are indicated by an arrow located below a nonspecific 50-kDa band. Hyg-1 for NIH3T3 and K562 cells contain the hygromycin-resistant gene only. **C:** Transient transfection assays. Cell lines stably transfected with ERK1 or ERK1-KR (dominant negative allele) were transiently transfected with Gal4-ELK1 and (Gal4)5-luciferase reporter plasmids, and treated with 50 nM PMA for 20 min at 37°C. Luciferase activity was determined as described under Materials and Methods. **D:** Cell survival analysis using colony formation assays. Untransfected NIH3T3 and K562 cells, cells transfected with hygromycin-selectable marker (Hygro-1 and -5 for NIH3T3 and Hyg-1 for K562), with wild-type ERK1 (ERK1–1 for NIH3T3 and ERK1–1, -4, and -5 for K562) or with ERK1-KR (ERK1-KR-4, -8, and -14 for NIH3T3 and ERK1-KR-1, -3, and -4 for K562) were heat shocked at 45°C for 40 min and plated for colony formation [Mivechi et al., 1994a]. Data points are means and s.e.m. of at least three independent experiments.

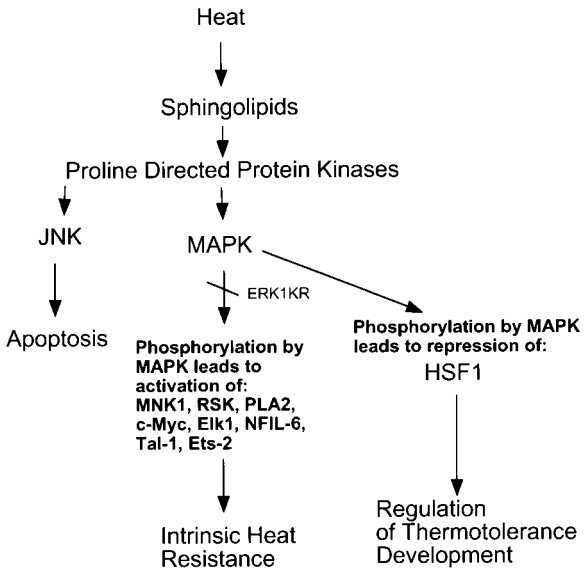

Fig. 8. Heat-shock activation of proline-directed protein kinases leads to the physiological and biochemical effects that are observed by heat. Blocking MAPK activation by ERK1-KR causes heat sensitivity of cells, suggesting that ERK1 may affect the intrinsic thermal sensitivity.

of damaged proteins and other cellular components, leading to increased intrinsic heat resistance. However, the detailed regulation of heat shock response through activation of these signaling pathways remains to be determined.

Taken together, our data support the view that ceramide is a key intermediate in stress-induced cell signaling events [Cuvillier et al., 1996; Hannun, 1996]. The metabolism of ceramide to sphingosine and sphingosine-1-phosphate activates MAPKs, which result in repair of damaged proteins, proliferation, and inhibition of apoptosis [Coroneos et al., 1996; Cuvillier et al., 1996]. If ceramide metabolism does not occur, ceramide itself activates JNKs predominantly via unknown intermediates and may induce cell cycle arrest and apoptosis [Coroneos et al., 1996; Cuvillier et al., 1996; Westwick et al., 1995].

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