Heat Shock Activates c-Src Tyrosine Kinases and Phosphatidylinositol 3-Kinase in NIH3T3 Fibroblasts*

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There is increasing evidence that cellular responses to stress are in part regulated by protein kinases, although specific mechanisms are not well defined. The purpose of these experiments was to investigate potential upstream signaling events activated during heat shock in NIH3T3 fibroblasts. Experiments were designed to ask whether heat shock activates p60 c-Src tyrosine kinase or phosphatidylinositol 3-kinase (PI 3-kinase). Using in vitro protein kinase activity assays, it was demonstrated that heat shock stimulates c-Src and PI 3-kinase activity in a time-dependent manner. Also, there was increased PI 3-kinase activity in anti-phosphotyrosine and anti-c-Src immunoprecipitated immunocomplexes from heated cells. Heat shock activated mitogen-activated protein kinase (MAPK) and p70 S6 kinase (S6K) in these cells. The role of PI 3-kinase in regulating heat shock activation of MAPK and p70 S6K was investigated using wortmannin, a specific pharmacological inhibitor of PI 3-kinase. The results demonstrated that wortmannin inhibited heat shock activation of p70 S6K but only partially inhibited heat activation of MAPK. A dominant negative Raf mutant inhibited activation of MAPK by heat shock but did not inhibit heat shock stimulation of p70 S6K. Genistein, a tyrosine kinase inhibitor, and suramin, a growth factor receptor inhibitor, both inhibited heat shock stimulation of MAPK activity and tyrosine phosphorylation of MAPK. Furthermore, a selective epidermal growth factor receptor (EGFR) inhibitor, tryphostin AG1478, and a dominant negative EGFR mutant also inhibited heat shock activation of MAPK. Heat shock induced EGFR phosphorylation. These results suggest that early upstream signaling events in response to heat stress may involve activation of PI 3-kinase and tyrosine kinases, such as c-Src, and a growth factor receptor, such as EGFR; activation of important downstream pathways, such as MAPK and p70 S6K, occur by divergent signaling mechanisms similar to growth factor stimulation.

The genes responsible for cellular responses to elevation of surrounding temperature are remarkably well conserved across all species from bacteria to mammals. In addition to elevated temperature, the induction of these genes and their encoded heat shock proteins is stimulated by other stressors, such as hypoxia or exposure to heavy metals, amino acid analogues, or viral infections. The production of heat shock proteins is thought to be a cellular protective response; these proteins act as molecular chaperones to facilitate folding and refolding of proteins denatured by environmental insults. Heat shock proteins have been implicated in a variety of medical conditions, from preservation of organs for transplantation, treatment of malignancies, or modulation of the immune system to the development of vaccines for infectious diseases (1).

The heat shock response has been intensively investigated in the effort to understand the regulation of heat shock gene transcription. In contrast, the molecular events by which the rise in temperature is transduced into cellular responses remain ill defined. There is increasing evidence that cellular stress responses are regulated by protein kinases, and indeed, heat shock activates a number of protein kinases, including p38/HOG1 kinase (2), Jun kinase (3), MAPK1 (4) and ribosomal S6 kinases (5). It is not known whether heat shock activates upstream signal molecules that regulate these downstream protein kinases.

Selective activation of gene transcription and protein translation are clearly important for cells to orchestrate an adaptive response to heat stress. MAPK and p70 S6K are critical regulators of gene transcription and protein translation, respectively, during cellular growth. It is known that mitogenic activation of MAPK requires Ras and Raf proteins, but activation of p70 S6K is independent of Raf and is probably dependent on activation of PI 3-kinase (6).

In the current study, we have investigated potential upstream signal events activated by heat shock and the signaling mechanisms that lead to heat shock activation of MAPK and p70 S6K in NIH3T3 cells. The results demonstrate that heat shock activates c-Src tyrosine kinase, PI 3-kinase, and the epidermal growth factor receptor (EGFR). In addition, the findings suggest that heat shock activates MAPK and p70 S6K via two distinct pathways.

MATERIALS AND METHODS

Myelin basic protein (MBP), H7, pertussis toxin, and genistein were purchased from Sigma; wortmannin was from Worthington Biochemical Co. (Freehold, NJ); and suramin was from Research Biochemicals (Natick, MA). [γ-32P]ATP (2000 Ci/mmol) and the ECL Western blotting detection kit were from Amersham Corp.; phosphatidylinositol was obtained from Avanti Polar Lipids Inc. (Alabaster, AL); and tyrphostin

* This work was supported in part by National Institute of Health Grant HL41315. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; hsp, heat shock protein; MAPAPK-FK-2, MAPK activated protein kinase-2; MBP, myelin basic protein; PI 3-kinase, phosphatidylinositol 3-kinase; S6K, S6 kinase; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; EGF, epidermal growth factor; EGFR, EGF receptor; HSF, heat shock transcription factor.
AG1478 was from Calbiochem. All other reagents of molecular biology grade were obtained from standard commercial sources.

Cell Culture—NIH3T3 and A431 cells were obtained from ATCC (Rockville, MD) and maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal bovine serum, 5% CO2 at 37 °C. Prior to harvest of cell lysates, cells were treated with heat shock for 5 min. Heat shock was conducted at 43 °C for 5 min on ice. The same technique was also used for the heat shock treatment of NIH3T3 cells used for the immunoprecipitation of c-Src (Santa Cruz Biotechnology).

Immunoprecipitation—After treatment, cells were rinsed with ice-cold phosphate-buffered saline containing 1 mM sodium orthovanadate. Cells were incubated with lysis buffer (1% Triton X-100, 25 mM Heps, pH 7.5, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 10 mM okadaic acid, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin for 15 min on ice. Insoluble material was pelleted by centrifugation. The lysate was incubated with an appropriate antibody for 2 h and then with 20 μl of protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The beads containing the immunoprecipitates were washed four times with lysis buffer and once with a kinase reaction buffer and then subjected to kinase activity assays.

In Vitro c-Src Activity Assay—Cell lysates were incubated with antibody against c-Src (Santa Cruz Biotechnology) and immunoprecipitated as described above. The washed immunocomplexes were resuspended in 40 μl of kinase buffer with 1 mM DTT, 40 μM ATP, and 1 μCi of [γ-32P]ATP and incubated at 30 °C for 10 min. The reaction was stopped by spotting an aliquot of the reaction mixture on phosphofilter paper, which was then washed extensively and radioactivity quantified by a scintillation counter.

Immunoblot of MAPK Phosphorylation—Cell lysates were prepared, and equal amounts of protein were suspended in loading buffer, heated at 95 °C for 5 min, resolved by gel electrophoresis, and visualized by Western blotting using anti-phosphorylated MAPK antibodies that recognize tyrosine-phosphorylated p44 ERK1 and p42 ERK2 (New England Biolabs, Beverly, MA) and an ECL detection system. The same membrane was then stripped and reblotted with an anti-ERK1 antibody (Santa Cruz Biotechnology).

In Vitro Assay of p70 S6K Activity—Cells were lysed and incubated with antibody against p70 S6K (2 μg/ml of protein, Santa Cruz Biotechnology) and immunoprecipitated as above. The washed immunocomplexes were resuspended in 40 μl of kinase buffer with 1 mM DTT, 40 μM ATP, 1 μCi of [γ-32P]ATP, and 10 μg of ribosomal S6 kinase substrate (Santa Cruz Biotechnology). The reaction mixture was incubated for 10 min at 30 °C. The reaction was stopped by spotting an aliquot of the reaction mixture on phosphofilter paper and then washed extensively and radioactivity quantified by a scintillation counter.

In Vitro Assay of MAPKAP-K2 Activity—Cells were lysed and incubated with antibody against MAPKAP-K2 (Upstate Biotechnology, Lake Placid, NY) and immunoprecipitated as above. The washed immunocomplexes were resuspended in 40 μl of kinase buffer with 1 mM DTT, 40 μM ATP, 1 μCi of [γ-32P]ATP, and 10 μg of hsp25 as substrate (StressGen Biotechnologies, Sidney, Canada). The reaction mixture was incubated for 10 min at 30 °C. The reaction was stopped by adding loading buffer and boiling for 5 min. The mixture was then separated by gel electrophoresis, and results were visualized by autoradiography.

Northern Blot of hsp70 mRNA—Cells were washed twice in cold PBS, and then RNA was extracted essentially as described by Chomczynski and Sacchi (10). Total RNA (20 μg) was fractionated by 1% agarose gel electrophoresis and transferred to a nylon filter. [32P]ATP labeled probes for hsp70 and β-actin were made by random primer labeling. After UV cross-linking, the filter was prehybridized in 50% formamide, 5 × saline/sodium phosphate/EDTA buffer, 5 × Denhardt’s solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA for 16–20 h at 42 °C. The filter was washed initially with 2 × SSC (1 × SSC is 0.15 NaCl and 0.015 M sodium citrate, pH 7.0, 0.1% SDS) and then with 0.1 × SSC, 0.1% SDS at 60 °C. The results were visualized by autoradiography and quantified by a PhosphorImager (Molecular Dynamics).

Immunoblot of EGF-R Phosphorylation—Equal amounts of protein extracts (500 μg) from control and heat-treated cells were immunoprecipitated with an EGFR antibody (Santa Cruz Biotechnology). Tyrosine phosphorylation of EGFR was visualized by Western blotting with an anti-phosphotyrosine antibody (P420, Transduction Laboratories) and an ECL detection system. The same membrane was then stripped and reblotted with the EGFR antibody to determine whether equal quantities of EGFR were immunoprecipitated.

RESULTS

Heat shock greatly increases protein tyrosine phosphorylation (11). Heat shock stimulation of MAPK activity correlates with increased phosphorylation of p42 and p44 MAPKs (4). We speculated that heat stress may activate upstream tyrosine kinases, which would then lead to phosphorylation of downstream protein kinases, such as MAPK. Src-family kinases have been implicated in signaling pathway of the UV stress response (12). We wondered whether heat shock may also activate c-Src. Using a Src-specific peptide substrate, c-Src activity was measured as described under “Materials and Methods.” We found that heat stress at 43 °C increased c-Src activity in a time-dependent manner (Fig. 1A). An in vitro autophosphorylation assay confirmed that heat shock increased c-Src activity (Fig. 1B).

Activation of Src-family tyrosine kinases may recruit binding of PI 3-kinase to the SRC homology 2 domain and stimulate PI 3-kinase activity (13). We wondered whether heat shock activation of c-Src in NIH3T3 cells was associated with activation of PI 3-kinase. In the first series of experiments, a general anti-phosphotyrosine antibody was used to immunoprecipitate cell lysates harvested following 5 min of heating because this time was the peak of activation of c-Src by heat shock (Fig. 1A). Heat shock greatly increased PI 3-kinase activity in these immunocomplexes (Fig. 2). There was also increased PI 3-kinase activity when lysates from heated cells were immunopre-
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Protein separation was achieved through SDS-polyacrylamide gel electrophoresis. Complexes were subjected to an autophosphorylation assay, and the products were immunoprecipitated with a c-Src antibody. Control (0 min) and heated samples (5 and 10 min) were used in this experiment showing the 60-kDa band representative of autophosphorylated c-Src. Control samples were measured by a scintillation counter and expressed as fold increase over control. The change in 3-kinase activity was presented as PIP 

**Fig. 1.** Heat shock activation of c-Src. Quiescent cells were heated at 43°C for 2, 5, and 10 min. Cell lysates (500 µg of protein) were immunoprecipitated with an antibody against c-Src. A, immunocomplexes were subjected to an in vitro kinase assay with a Src-specific peptide substrate. The degree of phosphorylation of the substrate was determined by a scintillation counter and expressed as fold increase over control samples ± S.E. (experiments were repeated three times). B, a representative autoradiogram of thin layer chromatography of PI 3-kinase was exposed for 24 h. The experiment was repeated once with similar results. 

**Fig. 2.** Heat shock activation of phosphotyrosine and c-Src-associated PI 3-kinase activity. Cells were heated at 43°C for 5 min or left untreated. Cell lysates (2 mg of protein) were immunoprecipitated with a phosphotyrosine (Anti-P-Tyrosine) or a c-Src antibody (Anti-c-Src). The immunocomplexes were then used to measure PI 3-kinase activity. Change in activity of PI 3-kinase is presented as production of phosphatidylinositol phosphate (PIP). The representative autoradiogram of thin layer chromatography of PI 3-kinase was exposed for 24 h. The experiment was repeated once with similar results. 

proteins were separated by SDS-polyacrylamide gel electrophoresis. Immunoprecipitation of PI 3-kinase with a specific c-Src antibody (Fig. 2). To confirm that heat shock activates PI 3-kinase, an antibody against the p85α subunit of PI 3-kinase was used to immunoprecipitate PI 3-kinase; we found that there was increased PI 3-kinase activity in these immunocomplexes (Fig. 3).

The heat shock response requires coordination of changes in transcriptional and translational rates of specific genes and proteins. For example, MAPK is a critical regulator of transcription, and p70 S6K has been shown to be important for protein synthesis. Heat shock activates MAPK and p70 S6K, which has been previously shown that heat stress also leads to phosphorylation of hsp25 after heat shock (Fig. 4). We confirmed that wortmannin inhibited this increase in p70 S6K activity in a dose-dependent manner, with an IC50 of ~10 nM (Fig. 4A). A next asked whether wortmannin also inhibited heat shock stimulation of MAPK. Much higher concentrations of wortmannin were required to inhibit heat-induced MAPK activity; there was approximately a 50% inhibition of MAPK activity at a wortmannin concentration of 100 nM (Fig. 4B). At this high concentration, wortmannin may have other effects, such as inhibition of myosin light chain kinase, so that its effects cannot be considered specific. It has been previously shown that heat stress also leads to phosphorylation of hsp25 by MAPKAPK-2 (14). We confirmed that observation and demonstrated that pretreatment with 100 nM wortmannin did not inhibit MAPKAPK-2-induced phosphorylation of hsp25 after heat shock (Fig. 4C).

Genistein inhibits tyrosine kinase activity in proteins such as c-Src and EGF receptors. To determine whether tyrosine kinases are involved in the activation of MAPK during heat shock, cells were pretreated with genistein for 1 h; the results demonstrated complete inhibition of heat shock activation of MAPK (Fig. 5A). Wortmannin had a partial inhibitory effect (Fig. 5A). In contrast, H7 (a protein kinase C inhibitor) had no effect on heat activation of MAPK. Down-regulation of protein kinase C with phorbol esters also did not inhibit heat shock activation of MAPK (data not shown). MAPKs are activated when they are phosphorylated at tyrosine and threonine residues. Using an anti-phosphotyrosine MAPK antibody, we confirmed that genistein inhibited tyrosine phosphorylation of MAPK by heat shock (Fig. 5B). The change in the extent of phosphorylation was not due to differential loading of proteins, as shown by Western blots with an anti-ERK1 antibody (Fig. 5C).

Further studies were done to investigate whether heat shock activation of MAPK and p70 S6K occurs by distinct signaling pathways. Growth factors, such as platelet-derived growth factor, activate p70 S6K without requiring Raf because overexpression of a dominant negative Raf mutant does not inhibit platelet-derived growth factor-induced activation of p70 S6K (15). In our experiments, we expressed a dominant negative Raf mutant (ΔRaf, a gift from L. T. Williams) that contains amino-terminal amino acid residues 1-257 of Raf1 without the kinase domain (16). ΔRaf inhibited heat shock stimula-
tion of MAPK activity (Fig. 6A) and tyrosine phosphorylation of MAPKs (Fig. 6B). However, the expression of N\(\Delta\)Raf did not inhibit heat-induced activation of p70 S6K activity, as measured using aliquots obtained from the same transfection experiments (Fig. 6C).

Heat shock activation of MAPK and p70 S6K appears to use signaling pathways reminiscent of those activated by growth factors. The surprising result that EGF receptors are involved in the cellular responses to UV irradiation suggests that growth factor receptors may play an important role in the signal transduction of stress responses (17). We investigated the hypothesis that growth factor receptors may also be involved in the heat shock response. Suramin is an extracellular antagonist of the platelet-derived growth factor receptor and may also inhibit other tyrosine kinase receptors (18). Cells were treated with 300 \(\mu\)M suramin for 30 min prior to heat shock and assayed for MAPK activity and tyrosine phosphorylation of MAPK in response to heat shock. We found that suramin inhibited heat activation of MAPK (Fig. 7, A and B). We also found that treatment of cells with suramin prior to heat shock partially inhibited heat shock-stimulated hsp70 mRNA accumulation (Fig. 7C).

To determine whether tyrosine kinases may have a role in regulating expression of heat shock protein mRNA, cells were pretreated with protein kinase inhibitors 2-aminopurine or genistein; both compounds completely inhibited heat shock induction of hsp70 expression in Northern blotting experiments. Heat shock induction of hsp70 mRNA expression was also partially inhibited by pretreatment with 100 nM wortmannin, although this may not be a specific effect of wortmannin (Fig. 7C).

Because the results from experiments with suramin suggest that growth factor receptor may be involved in the heat shock response, we investigated the possibility that heat treatment may cause cells to release growth factors into the medium. NIH3T3 cells were made quiescent in serum-free medium (overnight). The conditioned media from heat-treated (43 °C 30 min) were transferred to untreated but quiescent cells. Cells were incubated with the conditioned media for 2–30 min and then assayed for MAPK activity by immunocomplex kinase assay. We found that conditioned media from heat-treated cells did not induce an increase in MAPK activity (data not shown).

Although the experiments with conditioned media did not support the hypothesis that heat shock causes release of growth factors, there is increasing evidence that cellular...

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**Fig. 4.** Dose-response curve of wortmannin inhibition of p70 S6 kinase and MAPK. Cells were pretreated with wortmannin, a specific PI 3-kinase inhibitor at low nanomolar concentrations, prior to heat treatment at 43 °C for 30 min. A, cell lysates were immunoprecipitated with an antibody against p70 S6 kinase and used in an in vitro p70 S6 kinase assay described under “Materials and Methods.” The results were quantified by a scintillation counter and expressed as fold increase in activity over control (unheated). The data plotted are summarized from three separate experiments. B, cell lysates were immunoprecipitated with an antibody against ERK1 and used in an in vitro MAPK assay described under “Materials and Methods.” The data plotted are summarized from three separate experiments. C, cells were heated at 43 °C for 30 min with or without pretreatment with wortmannin (100 nm for 1 h). Cell lysates (500 \(\mu\)g of protein) were immunoprecipitated with an antibody against MAPKAPK-2 and used in an kinase assay with hsp25 as substrate. The experiment was repeated once with similar results.

**Fig. 5.** The tyrosine kinase inhibitor genistein blocked heat shock activation of MAPK. Cells were treated with 100 \(\mu\)M genistein, 100 nm wortmannin, or 100 \(\mu\)M H7 for 1 h prior to heating at 43 °C for 30 min. A, cell lysates were used in an in vitro MAPK assay with MBP as substrate; heat shock activation of MAPK was partially inhibited by wortmannin, completely inhibited by genistein, and not inhibited by H7. B, these results were confirmed by Western blots with an antibody against the tyrosine-phosphorylated MAPKs ERK1 and ERK2. C, Western blot with an anti-ERK1 antibody (cross-reacts with ERK2) shows equal loading of ERK proteins. All experiments were repeated at least three times with similar results.
stresses such as UV irradiation utilize growth factor receptors as a component of their cellular signaling (19). The EGFR appears to play a particularly important role in this intracellular signal cross-talk. Therefore, experiments were designed to investigate the hypothesis that EGFR may play a role in the heat shock activation of downstream signaling events. Tryphostin AG1478 is a selective EGFR inhibitor that inactivates the EGFR tyrosine kinase activity (20). Cells were preincubated with AG1478 for 1 h prior to heat treatment at 43 °C for 30 min. MAPK activation was monitored by Western blots using a specific antibody against anti-phosphotyrosine MAPK antibody as described earlier. We found that AG1478 completely inhibited heat shock activation of MAPK even more effectively than suramin (Fig. 8A). Although AG1478 minimally inhibited heat induction of MAPK-2 activity, this is probably due to a nonspecific effect of AG1478 because stress activation of the p38/MAPK-2 pathway is thought to be in a separate pathway from the MAPK pathway. Not surprisingly, suramin also did not block heat shock activation of MAPK-2 (Fig. 8B).

To test the hypothesis that EGFR is involved in the heat shock activation of MAPK, NIH3T3 cells were stably transfected with a dominant negative EGFR mutant and wild-type human EGFR (HERCD533 and HER1, respectively; gifts from A. Ullrich). The HERCD533 mutant lacks the cytoplasmic kinase domain and therefore inhibits EGFR downstream signaling by formation of signaling-defective heterodimers with the wild-type receptor (20). NIH3T3 cells expressing HERCD533 when heat shocked had attenuated MAPK activation compared with wild-type cells and with cells stably transfected with HER1 (Fig. 8C). This result suggests that EGFR plays an important role in heat shock activation of downstream intracellular events.

Upon activation, EGFR is tyrosine autophosphorylated at tyrosine residues. To determine whether heat shock activates EGFR, we used A431 cells, which express high levels of endogenous EGFR. Equal amounts of protein extracts from control and heat-shocked A431 cells were immunoprecipitated with an antibody against ERK1 and used in a MAPK assay with MBP as substrate (A) or used in Western blots with an anti-phosphotyrosine MAPK antibody (B). C, in vitro p70 S6 kinase assay with an RSK-specific peptide substrate. The results demonstrate that heat shock activation of MAPK occurs via Raf, but activation of p70 S6 kinase is independent of Raf. All experiments were repeated at least three times.

![Fig. 6](image6.png)

**Fig. 6.** Dominant negative Raf (NΔRaf) inhibits heat shock activation of MAPK but not p70 S6 kinase. Cells were transfected with NΔRaf or empty expression vector. Cells were heated at 43 °C for 30 min or left untreated. Cell lysates were either immunoprecipitated with an antibody against ERK1 and used in a MAPK assay with MBP as substrate (A) or used in Western blots with an anti-phosphotyrosine MAPK antibody (B). C, in vitro p70 S6 kinase assay with an RSK-specific peptide substrate. The results demonstrate that heat shock activation of MAPK occurs via Raf, but activation of p70 S6 kinase is independent of Raf. All experiments were repeated at least three times.

![Fig. 7](image7.png)

**Fig. 7.** Growth factor antagonist suramin blocks heat shock activation of MAPK and hsp70 mRNA expression. Cells were treated with 300 μM suramin for 1 h prior to heat treatment. A, cell lysates were used in an in vitro MAPK assay; bands shown are phosphorylated substrate (MBP corresponds to MAPK activity). Suramin inhibited heat shock activation of MAPK. B, this inhibition was confirmed by Western blots with an antibody against tyrosine-phosphorylated MAPKs. The bands shown are tyrosine-phosphorylated ERK1 and ERK2. C, cells were treated with 300 μM suramin, 100 μM 2-aminopurine (2AP), 100 μM genistein, or 100 nM wortmannin for 1 h prior to heating at 43 °C for another hour. Cells were harvested to obtain mRNA and used in Northern blots with an hsp70 probe. The bands correspond to hsp70 mRNA abundance. D, the same membrane was stripped and hybridized with a β-actin probe to show equal loading of mRNA. All experiments were repeated once with similar results.
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A

CT HS AG SUR

P-ERK1 P-ERK2

B

CT HS AG SUR

HSP25

C

WT CT HS HER CD53 EGFR

P-ERK1 P-ERK2

Fig. 8. Inhibition of EGFR suppresses heat shock activation of MAPK. Cells were treated with tryphostin AG1478 (50 μM) (AG) or suramin (300 μM) (SUR) for 1 h prior to heat shock (HS). A, cell lysates (100 μg of protein) separated by SDS-polyacrylamide gel electrophoresis and tyrosine-phosphorylated MAPK detected by a specific anti-phospho-MAPK antibody. B, samples from the same experiment were also assayed for MAPKAPK-2 activity with an immunocomplex kinase assay. C, NIH3T3 cells stably transfected with human EGFR (HER) or dominant negative mutant (CD53) or wild type were either left untreated or heat shocked at 45 °C for 30 min and then assayed for tyrosine-phosphorylated MAPK species (left panel). Cells were also treated with EGF (100 ng/ml) and assayed for phosphorylated ERKs (right panel). The membranes were also stripped and rebotted with an ERK1 antibody showing equal loading of MAPK proteins (data not shown). The experiment was repeated once with similar results.

Fig. 9. Heat shock induces tyrosine phosphorylation of EGFR. A431 cells heated at 43 °C for 30 min were lysed and immunoprecipitated with an antibody against EGFR and then blotted with an anti-phosphotyrosine antibody, PY20 (top panel). The same membrane was then stripped and rebotted with the EGFR antibody (bottom panel). The experiment was repeated once with similar results.

DISCUSSION

The heat shock response is a rapid, highly regulated adaptive response to stress, and it involves coordinate control of multiple signal transduction pathways. The complexity of the cellular heat shock response is quite apparent, and heat shock activates multiple kinase (JNK/SAPK, p38/HOG1, and ERKs) cascades. However, some of the earliest signal transduction events after heat shock appear to occur near the cell membrane. We demonstrated that heat shock activates c-Src, a kinase known to be anchored to the inner surface of cell membrane by a amino-terminal myristic acid moiety (15). Heat shock activation of c-Src is a relatively early event and is probably associated with activation of PI 3-kinase as indicated by our result showing increased PI 3-kinase activity in anti-c-Src immunocomplexes.

Like Src family kinases, PI 3-kinase is located near the cell membrane and is associated with other signal molecules through its SRC homology 2 and SH3 domains (21). Heat shock activation of PI 3-kinase may involve other tyrosine-phosphorylated proteins because there is increased PI 3-kinase activity in anti-phosphotyrosine immunocomplexes. One possible candidate may be receptor tyrosine kinases. This hypothesis is supported by our results indicating that the signal pathways used by heat shock to activate MAPK and p70 S6 kinase resemble those used by growth factors, and heat shock activation of MAPK is blocked by suramin. Protein perturbation caused by hyperthermia may in some way stimulate cell surface receptor tyrosine kinases to initiate the downstream signaling events. Indeed, inhibition of heat shock activation MAPK by tryphostin AG1478 and a dominant negative EGFR mutant strongly suggests that EGFR may be involved in the heat shock response. These results are reminiscent of other cellular stress responses, such as UV and oxidative stress, both of which stimulate tyrosine phosphorylation of EGFR (22, 23).

To confirm that EGFR is involved in the heat shock response, we used A431 cells, which express high levels of EGFR compared with NIH3T3 cells, to determine whether heat stress induces tyrosine phosphorylation of EGFR. Our results indicate that heat shock strongly induces tyrosine phosphorylation of EGFR. The results of the experiments using suramin to inhibit heat shock activation of MAPK are compatible with the possibility that heat shock may induce cells to release EGF into the medium. However, the conditioned media from heat-treated cells did not induce an increase in MAPK activity, and pretreatment of cells with an neutralizing antibody against EGFR also did not block heat shock-induced phosphorylation of EGFR but, as expected, inhibited EGF-induced receptor phosphorylation. These results suggest that EGFR is not being released by the heat-stressed cell, leading to activation of EGFR and MAPK in an autocrine fashion.

In NIH3T3 cells, EGFR activation does not lead to activation of PI 3-kinase (24), but Src can activate PI 3-kinase (21) and phosphorylate EGFR (25). It is possible that the initial event in the heat shock response is Src activation, which leads to phosphorylation of EGFR and activation of MAPK; via a separate pathway, it also leads to activation of PI 3-kinase and subsequent activation of p70 S6 kinase. Further investigation is needed to clarify the possible interactions between activation of Src, EGFR, and PI 3-kinase following heat shock.

The role of p70 S6 kinase during heat shock is unclear; however, regulation of protein synthesis is obviously important during the heat shock response because there is a general shutdown of protein synthesis but a selective increased production of heat shock proteins. Heat shock activation of p70 S6 kinase is probably via PI 3-kinase because it was highly sensitive to wortmannin inhibition. This result is consistent with findings that growth factor activation of p70 S6 kinase is also via PI 3-kinase (15). PI 3-kinase has also been implicated in regulation of cytoskeletal changes via a separate pathway from p70 S6 kinase (6).

Cellular stress, including heat shock, also activates a distinct stress pathway, leading to stimulation of p38 kinase, which activates MAPKAPK-2, which subsequently phosphorylates hsp25 (14). The role of phosphorylated hsp25 is controversial; it may have cellular protective effects by preventing stress-induced cytoskeletal damages (26). Our observation that heat shock activation of MAPKAPK-2 is insensitive to wortmannin shows that this is a separate signal pathway from those used by heat shock to activate p70 S6 kinase.

The key regulatory event in initiating transcription of heat shock genes is binding of a heat shock transcription factor (HSF-1) to a heat shock consensus element found in the promoters of all heat shock genes (27). Heat shock activates HSF-1 by promoting its trimerization, nuclear localization, and increased DNA binding capacity (28). Studies have indicated that binding of HSF1 to the heat shock consensus element is not sufficient for full transcription of heat shock genes, and other
regulatory events involving protein phosphorylation appear to play a role (29). Heat shock induces hyperphosphorylation of HSF-1, but the protein kinases that are involved in phosphorylating HSF-1 are yet to be identified. Our data confirm that protein kinases play an important role in regulating expression of heat shock protein because the protein kinase inhibitors genistein and 2-aminopurine completely inhibited heat shock induction of hsp70 mRNA expression. Chu et al. (30) showed that MAPK, in conjunction with glyoxen synthase kinase 3, phosphorylates heat shock factor-1 and represses transcriptional activation of the heat shock protein 70 promoter. This result suggest that MAPK may serve as a negative regulator of heat shock protein production and deactivate the heat shock response during recovery from stress. Activation of MAPK appears to play a protective role during cellular stress. Oxidative stress activates MAPK, and inhibition of MAPK activation decreases cell survival after oxidative stress (31). It is possible that activation of MAPK by heat shock may have similar protective effects in this setting.

Further studies will be required to clarify how activation of signal pathways at the cell membrane surface interact with the key regulatory events occurring in the nucleus during heat shock. It will also be important to determine the physiological role of the MAPK and p70 S6 kinase in the heat shock response. Investigations in this area will lead to a better understanding of this fundamental and fascinating cellular response to cellular stress.

Acknowledgments—HERCD533 and HER1 were gifts from A. Ullrich (Max Planck Institute), and NARaf was a gift from L. T. Williams (University of California, San Francisco).

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