Mild heat shock induces cyclin D1 synthesis through multiple Ras signal pathways

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Accepted 16 January 2002

First published online 26 February 2002

Edited by Richard Marais

Abstract Hyperthermia such as that occurring during fever may improve cell survival during infection, although its mechanism of action is largely unknown. Here we show that acute exposure to mild, but not severe, heat shock induces the synthesis of cyclin D1 that plays a critical role(s) in G1 progression of the cell cycle. This induction seemed to be regulated through multiple Ras signal pathways involving extracellular signal-regulated kinase, phosphatidylinositol 3-kinase, and Rac1/NADPH oxidase, all of which have well been documented to be responsible for growth factor-induced cyclin D1 expression. In a physiological sense, mild heat shock may regulate cell proliferation through inducing cyclin D1 along with growth factors.

Key words: Heat shock; Cyclin D1; Ras; Rac1

1. Introduction

Since its discovery in 1962 by Ritossa [1], the heat shock response has been extensively studied by a number of investigators to understand the molecular mechanism underlying the cellular response to heat stress. In most cases, heat shock has been thought to act as a proteotoxic stress that causes protein denaturation in cells and exerts a variety of anti-proliferative effects in mammalian cells. Acute exposure to heat shock leads to a transient arrest of cells at mainly two cell cycle check points, the G1/S and G2/M transitions, through inducing $p21^{WAF1}$ cyclin-dependent kinase (CDK) inhibitor and other regulatory proteins [2–7]. More severe heat shock also leads to the programmed cell death known as apoptosis by activating p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase [8–10]. The heat shock stress response has been evaluated with relatively high temperature. However, hyperthermia such as that occurring during fever may be beneficial to host during infection [11–13], although its molecular mechanism is not clearly understood. Recently, we have demonstrated that mild, but not harsh, heat shock response is controlled by a small GTPase Rac1 [14]. Since Rac1 has been implicated in growth factor-mediated cell proliferation through activating various downstream signal molecules [15–19], we suggest that mild heat shock may exert positive roles in cell growth.

When quiescent cells enter the cell cycle in response to mitogenic signals, they induce genes encoding D-type cyclins (D1, D2, and D3), key molecules required for passage through the restriction point in the mammalian cell cycle [20–22]. The cyclins assemble with their catalytic partners, CDK4 and CDK6, as cells progress through G1 phase, thereby inactivating the growth-suppressive function of retinoblastoma protein through its phosphorylation [23–25]. Cyclin D-CDK4/6 complex also titrates CDK inhibitors, such as $p27^{Kip1}$ and $p21^{Cip1}$, facilitating cell cycle progression [23]. Growth factor-induced cyclin D1 expression is mainly regulated by multiple Ras signal pathways which involve (1) the Raf/MAPK kinase/extracellular signal-regulated kinase (ERK) 1 and 2 pathways, (2) the phosphatidylinositol (PI) 3-kinase/Akt (PKB) pathway, and (3) the Rac1/NADPH oxidase/reactive oxygen species (ROS) pathway [17,18,26–30]. The level of cyclin D1 is also post-transcriptionally controlled by the PI 3-kinase/Akt (PKB) pathway. Akt (PKB) phosphorylates and inactivates glycogen synthase kinase-3β (GSK-3β), which can phosphorylate cyclin D1 and stimulates its nuclear export, and accelerates its ubiquitin-dependent proteosomal degradation in the cytoplasm [17,18,26,27,31,32]. Furthermore, GSK-3β is involved in targeting the adenomatous polyposis coli-mediated degradation of β-catenin, which regulates the expression of cyclin D1 [17,18,26,27,33,34]. p38MAPK, which is induced by several different kinds of stresses, has also been implicated in down-regulation of cyclin D1 by regulating its transcription and degradation [35,36].

In this study, we investigated if heat shock could exert stimulatory effects on cell proliferation. Here we demonstrate that mild, but not severe, heat shock induces the synthesis of cyclin D1 through multiple Ras signal pathways involving ERK, PI 3-kinase, and Rac1/NADPH oxidase, indicating that physiological mild heat shock may participate in the regulation of cell proliferation along with growth factors.

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Abbreviations: ERK, extracellular signal-regulated kinase; PI 3-kinase, phosphatidylinositol 3-kinase; CDK, cyclin-dependent kinase; GSK-3β, glycogen synthase kinase-3β; MAPK, mitogen-activated protein kinase; HSP, heat shock protein

0014-5793/02/$22.00
PII: S0014-5793(02)02459-6

2. Materials and methods

2.1. Cell culture, serum starvation and drug treatment

NIH3T3, Rat-2, and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a 37°C humidified incubator with 5% CO_2. For serum starvation, cells cultured in the complete growth media were either changed to serum free-DMEM and incubated for 24 h or trypsinized and plated in 0.5% FBS–DMEM and incubated for 24 h. Inhibitors including cycloheximide (100 μM), actinomycin D (5 μg/ml), LY294002 (10 μM), wortmannin (100 nM), diphenyleneiodonium (DPI; Sigma Chemical Co.), PD98059 (30 μM) and SB203580 (30 μM, Calbiochem), were pretreated 1 h before exposure to heat shock or 10% FBS.

2.2. Transfection and protein analysis

Transfection of Rac1N17 and Western blotting with antibodies to cyclin D1, cyclin A (Santa Cruz Biotechnology), heat shock protein (HSP)70 (StressGen Biotechnologies Corp.), phospho-GSK-3β, GSK-3β, phospho-ERK, ERK, phospho-p38MAPK and p38MAPK (Calbiochem) were performed as described previously [14]. For analysis of Ras and Rac1 activation, we used Ras and Rac activation assay kit (Upstate Biotechnology) designed to detect for the binding activity of GTPases to downstream effector molecules.

2.3. Confocal microscopy

Rat-2 cells were grown on round coverslips in multiwell culture plates and exposed to either epidermal growth factor (EGF, 100 ng/ml) or heat shock for the times indicated. The cells were fixed with 3.7% (w/v) formaldehyde, and then permeabilized with 0.2% Triton X-100. The cells were treated with 0.165 M NBD-phallacidin for actin staining and observed under a confocal microscope (LSM510, Carl Zeiss).

3. Results

3.1. Mild heat shock induces the expression of cyclin D1

To investigate the effects of heat shock on cell cycle regulation, we treated quiescent serum-starved NIH3T3 and Rat-2 cells with different heat shock temperatures (39–45°C for 40 min) and examined the levels of cyclin D1, a key molecule required for passage through the restriction point in the mammalian cell cycle [20,21]. As demonstrated by other investigators [17,18,20–22,26,27], quiescent cells contain low levels of cyclin D1 and serum stimulates its induction (Fig. 1A). Exposing these cells to heat shock conditions in a range of 39–43°C increased the cyclin D1 level in temperature- and time-dependent manners (Fig. 1A). In contrast, severe heat shock treatment at 45°C did not enhance cyclin D1 synthesis, indicating that cyclin D1 expression is only triggered by mild heat shock (Fig. 1A). Similar results were obtained from other mammalian cells such as HeLa cells (data not shown), suggesting the ubiquitous cyclin D1 induction by mild heat shock. While serum stimulation of quiescent cells maintained the increased levels of cyclin D1, heat shock transiently increased its levels, with the maximal induction at 9 h after heat shock at 42°C for 40 min (Fig. 1B) and at 16 h after prolonged exposure to 39.5°C (Fig. 1C). Exposure of quiescent cells to mild heat shock or serum induced cyclin A (Fig. 1C), which is required for DNA synthesis and begins to be synthesized as cells approach the G1-S transition [37,38]. Cyclin A induction was evident 16 h after stimulation with either mild heat shock or serum.

3.2. Heat shock itself acts as a signal for the cyclin D1 expression

Heat shock has been shown to induce secretion of growth factors such as fibroblast growth factor in animal cell cultures [11,12,39], even though in an inactive form. Therefore, we tested the conditioned media (collected from cultures heat-shocked at 42°C for 40 and 80 min and recovered at 37°C for 6 h) for its ability to induce the cyclin D1. However, no significant induction was observed, indicating that heat shock itself acts as a signal activator for the cyclin D1 induction (Fig. 2A).

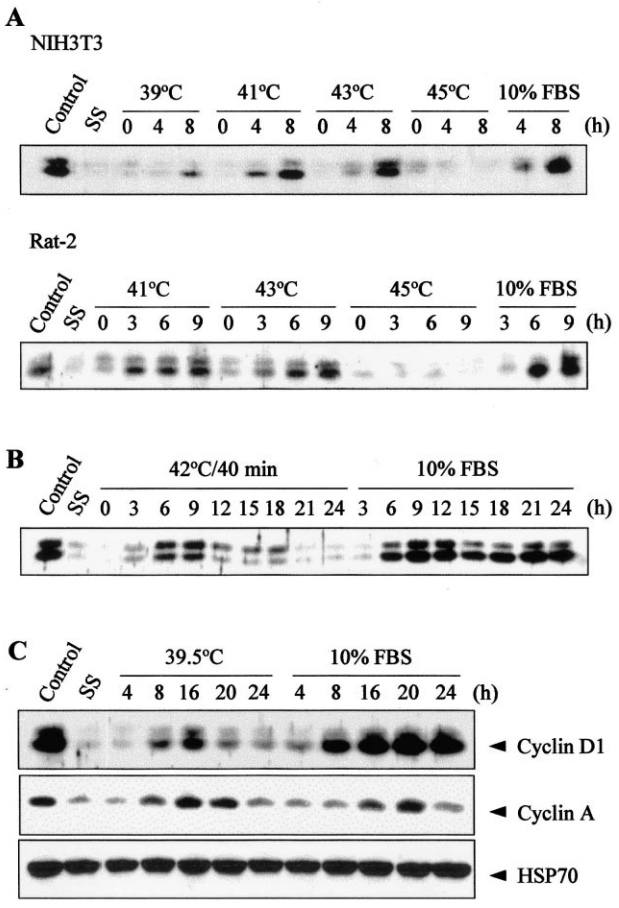

Fig. 1. Induction of cyclin D1 by mild heat shock. A: Serum-starved (SS) NIH3T3 and Rat-2 cells were heat-shocked at either 39, 41, 43 or 45°C for 40 min and then allowed to recover at 37°C for the times indicated. In parallel, serum-starved cells were treated with 10% FBS. Cyclin D1 levels were determined by immunoblotting with anti-cyclin D1. B,C: Transient induction in cyclin D1 levels in NIH3T3 cells that were either heat-shocked at 42°C for 40 min and recovered at 37°C for the times indicated (B) or heat-shocked at 39.5°C for the times indicated (C) and the lysates analyzed by immunoblotting with antibodies to cyclin D1, cyclin A, and HSP70.

3.3. Heat shock induction of cyclin D1 is regulated at transcriptional and translational levels

The expression of cyclin D1 in response to serum stimulation is regulated at transcriptional levels by several different types of transcriptional factors including Ets and AP1 [17,18,26,27]. The treatment of cycloheximide and actinomycin D prevented the induction of cyclin D1 in response to serum and mild heat shock (Fig. 2B), suggesting the regulation of heat shock-induced cyclin D1 expression at transcriptional and translational levels.

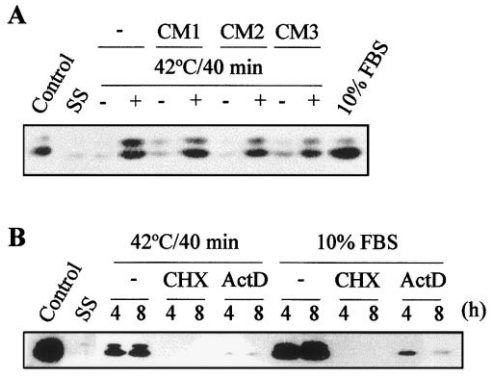

Fig. 2. Heat shock itself acts as a signal for the cyclin D1 expression at both transcriptional and translational levels. A: Effects of the conditioned media on the levels of cyclin D1. The conditioned media were collected from cultures that were serum-starved for 24 h and either incubated at 37°C for 6 h (CM1) or heat-shocked at 42°C for 40 min (CM2) and 80 min (CM3) and recovered at 37°C for 6 h. Then, serum-starved cells were treated with the conditioned media and either incubated at 37°C or heat-shocked at 42°C for 40 min and recovered at 37°C for 8 h. B: Effects of cycloheximide (CHX) or actinomycin D (ActD) on cyclin D1 induction by heat shock. Cycloheximide (100 μM) and actinomycin D (5 μg/ml) were pretreated 1 h before exposure to heat shock or 10% FBS.

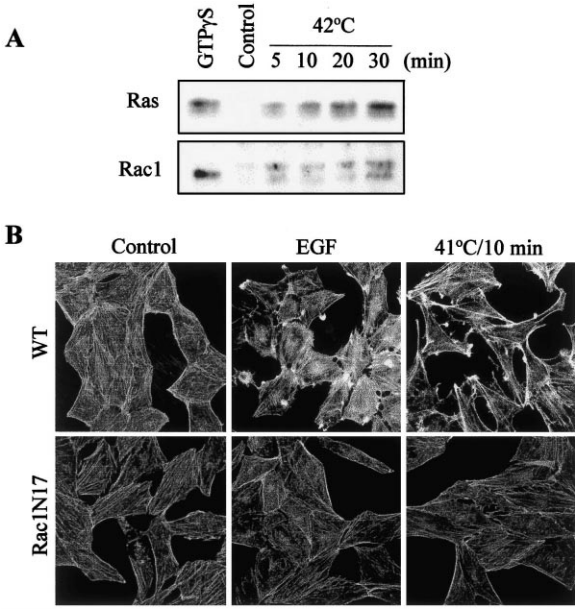

Fig. 3. Activation of Ras and Rac1 GTPases by mild heat shock. A: Heat shock-induced activation of Ras and Rac1. Serum-starved NIH3T3 cells were heat-shocked at 42°C for the times indicated. The cellular polypeptides were analyzed for Ras or Rac1 activation by a pull-down assay using Raf-RBD (top) or PAK-RBD-conjugated bead (bottom), respectively. GTPγS, in vitro stimulation by addition of 100 μM GTPγS to the control cell extracts. B: Inhibition by Rac1N17 of heat shock-induced membrane ruffling. Rat-2 wild type (WT) and Rac1N17 cells were serum-starved for 24 h and exposed to EGF (100 ng/ml) or heat shock (41 °C) for 10 min, fixed, permeabilized, and stained with 0.165 μM NBD-phallacidin and observed under a confocal microscope (×400, Carl Zeiss, LSM510).

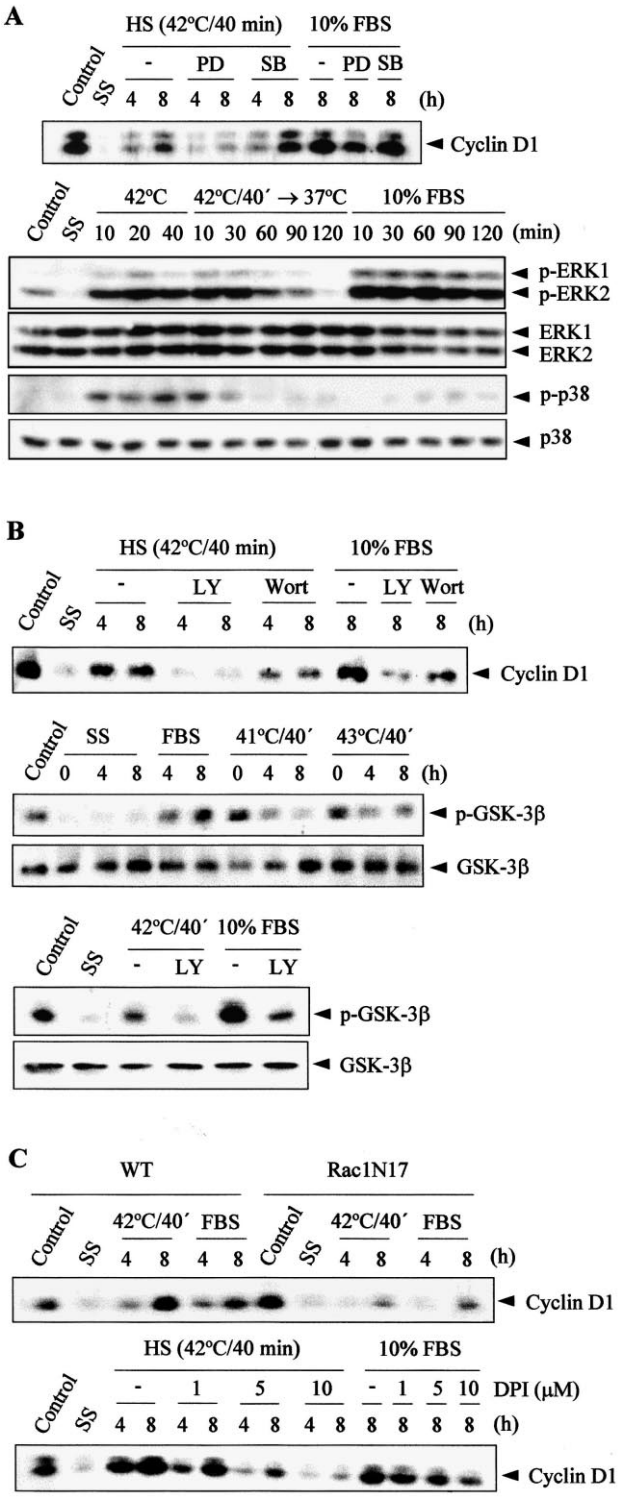

Fig. 4. Heat shock induction of cyclin D1 is dependent on multiple Ras signal pathways. A: Effects of PD98059 (PD) and SB203580 (SB) on heat shock-induced cyclin D1 expression (top) and activation of ERK1/2 and p38MAPK by heat shock (lower panels). B: Inhibition by LY294002 (LY) and wortmannin (Wort) of heat shock-induced cyclin D1 expression and PI 3-kinase-dependent phosphorylation of GSK-3β by heat shock. C: Inhibition by Rac1N17 and DPI of heat shock-induced cyclin D1 expression. PD98059 (30 μM), SB203580 (30 μM), LY294002 (10 μM), and wortmannin (100 nM) DPI were pretreated 1 h before exposure to heat shock or 10% FBS.

3.4. Heat shock activates several components of the Ras signal pathways

Cyclin D1 expression is mediated mostly by multiple Ras signal pathways [17,18,26,27]. Therefore, we examined possible involvement of the Ras pathways in the mild heat shock-induced cyclin D1 expression. Here we show that several components of the Ras signal pathways are activated. Mild heat shock appeared to activate Ras as evidenced by a pull-down assay using the Ras binding domain of Raf (Fig. 3A). Previously, we demonstrated that Rac1, a downstream molecule of the Ras pathways, is activated in response to heat shock [14]. Mild heat shock-induced Rac1 activation was also confirmed by a pull-down assay using the Rac1 binding domain of PAK (Fig. 3A). In addition, mild heat shock-induced membrane ruffling [14] was prevented by Rac1N17, a dominant negative mutant of Rac1 (Fig. 3B). Furthermore, mild heat shock activated ERK1/2 through its phosphorylation at Thr-202/Tyr-204, but transiently compared to that of serum-stimulated cells (Fig. 4A). It is notable that heat shock induces phosphorylation of p38MAPK at Thr-180/Tyr-182 that is involved in the down-regulation of cyclin D1, while serum addition does not. We further show that heat shock induces phosphorylation of GSK-3β at Ser-9, a well documented Akt (PKB) phosphorylation site that is inactivating modification of GSK-3β (Fig. 4B). LY294002, a PI 3-kinase inhibitor, significantly blocked the phosphorylation of GSK-3β in response to heat shock (Fig. 4B), suggesting the PI 3-kinase-Akt/PKB-GSK-3β pathway activation. The GSK-3β phosphorylation by heat shock was transient (detected only in cells heat shocked at 41–43°C for 40 min, but not in cells recovered at 37°C) compared to that induced by serum.

3.5. Mild heat shock-induced cyclin D1 expression is prevented by inhibition of the Ras signal pathways

We also examined if inhibition of the Ras signal pathways prevented mild heat shock-induced cyclin D1 expression. Inhibition of ERK with the MEK1 inhibitor PD98059 partially prevented the induction of cyclin D1, whereas the p38MAPK inhibitor SB203580 enhanced (Fig. 4A), in agreement with previous observations of the positive and negative roles of ERK and p38MAPK, respectively [17,18,26,27,35,36]. Two structurally distinct inhibitors of PI 3-kinase, LY294002 and to a lesser extent, wortmannin, prevented mild heat shock-induced cyclin D1 expression (Fig. 4B). In addition, cyclin D1 expression by heat shock was significantly reduced by the dominant negative mutant (N17) of Rac1 (Fig. 4C). Furthermore, DPI, a potent NADPH oxidase inhibitor (Fig. 4C), and *N*-acetyl-L-cysteine (data not shown), a ROS scavenger, also prevented the cyclin D1 induction. These results suggest that mild heat shock-induced cyclin D1 expression is mediated by multiple Ras signal pathways involving ERK1/2, PI 3-kinase/Akt (PKB)/GSK-3β, and Rac1/NADPH oxidase.

4. Discussion

In this study we show that heat shock induces the synthesis of cyclin D1 that plays a critical role(s) for passage through the restriction point. This is the first report of heat shock induction of cyclin D1. A number of investigators have previously demonstrated that heat shock reduces the levels of cyclin D1 in mammalian cells and yeast, which arrests the cells mostly at the G1 stage of the cell cycle [5–7]. This discrepancy may be due to the differences between temperatures used in the experiments. In support with this idea, we show that harsh heat shock (for example, 45°C for 40 min) does not stimulate the synthesis of cyclin D1 although mild heat shock increases its level (Fig. 1). It has been proposed that the effects of heat shock are determined by both temperature and exposure time: as temperature increases by 1°C, the time required for the same extent of the heat shock response is reduced by two-fold [40]. Thus, a mild heat shock response can be obtained by decreasing either the temperature or exposure time.

Several lines of evidence revealed that mild heat shock-induced cyclin D1 expression is mediated by multiple Ras effector pathways. First of all, heat shock activated several components of the Ras signal pathways including Ras, Rac1 (Fig. 3), and ERK1/2 (Fig. 4A). It has also been shown to activate PI 3-kinase [41] and Akt (PKB) [42]. Furthermore, heat shock induces phosphorylation at Ser-9 and possible inactivation of GSK-3β, which can be prevented by LY294002, a PI 3-kinase inhibitor (Fig. 4B). Thus, heat shock is likely to activate the PI 3-kinase-Akt/PKB-GSK-3β pathway. PI 3-kinase may act both upstream and downstream of Rac1 [17,18,26,27]. Secondly, inhibition of the Ras signal pathways significantly abolished heat shock-induced cyclin D1 expression (Fig. 4). PD98059 (a MEK1 inhibitor), LY294002 and wortmannin (PI 3-kinase inhibitors), a dominant negative mutant Rac1N17, and DPI (a potent NADPH oxidase inhibitor) prevented the cyclin D1 induction in response to heat shock as well as serum (Fig. 2). These results suggest that mild heat shock-induced cyclin D1 expression is mediated through multiple Ras signal pathways involving ERK1/2, PI 3-kinase/Akt (PKB)/GSK-3β, and Rac1/NADPH oxidase, which are responsible for growth factor-induced cyclin D1 expression.

It is not clear how heat shock activates the Ras signal pathway. Heat shock may activate multiple growth factor receptors including EGF receptor tyrosine kinase [42] by affecting membrane structure and mobility [43], which in turn activates the Ras signal pathway. Otherwise, the Ras molecule may be directly activated by mild heat shock.

While mild heat shock induction of cyclin D1 is regulated mostly at the transcriptional and translational levels, its turnover may also be controlled by heat shock. Since heat shock induces the phosphorylation and inactivation of GSK-3β (Fig. 4B), which stimulates degradation of cyclin D1 as well as β-catenin that regulates the expression of cyclin D1 [17,18,26,27,31–34], cyclin D1 degradation could be inhibited by heat shock.

Compared to prolonged cyclin D1 induction by serum stimulation, mild heat shock-induced cyclin D1 expression was transient. For example, acute exposure to mild heat shock at 41–43°C for 40 min results in cyclin D1 induction with a maximal level at 9 h after heat shock and a decline thereafter (Fig. 1B). Transient ERK1/2 activation (Fig. 4A) and GSK-3β inactivation (Fig. 4B) may explain this transient cyclin D1 induction in response to heat shock. p38MAPK may also be involved in this phenomenon (Fig. 4A). When cells are exposed to prolonged heat shock at 39.5°C, the cyclin D1 induction was sustained for a longer period (until 16 h after heat shock) (Fig. 1C). In addition, prolonged exposure to mild heat shock was sufficient for the induction of cyclin A that is required for DNA synthesis and begins to be synthesized as cells approach the G1-S transition (Fig. 1C). The G0-arrested cells pass the restriction point 14–16 h after the ad-

dition of growth factors, and enter S-phase 6–8 h after [17,18]. However, mild heat shock did not exert significant effects on cell proliferation, although it caused cyclin D1 to assemble with CDK4/6 and to translocate to nucleus (data not shown). These results suggest that physiological mild heat shock may facilitate growth factor-stimulated cell proliferation through inducing cyclin D1, while severe heat shock leads to the cell cycle arrest [2–7] and apoptosis [8–10]. It was recently demonstrated that mild heat shock stimulates cell proliferation and DNA synthesis in human bone marrow stromal cells and Mg-63 cells in vitro [44]. It is possible that mild heat shock may activate other molecules that stimulate cell proliferation and inhibit serum deprivation-induced apoptosis. In fact, heat shock activates a number of molecules that promote cell survival including Ras, Rac1, ERK1/2, PI-3 kinase, and Akt, but inactivates GSK-3β that is involved in programmed cell death, even though transiently compared to that by serum stimulation (Figs. 3 and 4).

Body temperature in mammals including human is finely regulated in a homeostatic manner. When human becomes febrile, body temperature increases only by 1–2°C, which can be considered as a mild heat shock. Temperature elevation has been suggested to constitute a beneficial component of effective host defense, by directing killing or inhibiting growth of invading microbial pathogens, by stimulating expression of pathogen HSPs, potent activators of host immune defenses, by inducing cytoprotective HSPs in host cells, and by modifying and optimizing the host immune response [11–13]. For instance, mild heat shock facilitates interleukin 1-dependent T-cell proliferation and activation. Our results may provide new insights into the elucidation of molecular mechanism for body temperature-dependent growth of organisms including plants, animals, and human.

Acknowledgements: This work was supported by the Korea Research Foundation Grant (KRF-99-0005-F00037). We thank Drs. J.S. Kim and D.Y. Moon for critical review of this manuscript.

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