ASBMB
JOURNAL OF LIPID RESEARCH

Critical role of p42/44^{MAPK} activation in anisomycin and hepatocyte growth factor-induced LDL receptor expression: activation of Raf-1/MEK-1/p42/44^{MAPK} cascade alone is sufficient to induce LDL receptor expression

Punita Dhawan, April Bell, Amit Kumar,[1] Carmen Golden, and Kamal D. Mehta[2]

Department of Biochemistry and Molecular Biology, College of Medicine, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, AR 72205

Abstract The protein synthesis inhibitor anisomycin activates stress-related mitogen-activated protein kinases (MAPKs), namely, c-jun NH_2-terminal kinase (p46/54^{JNK}) and p38^{MAPK} in mammalian cells. In this paper, we show that although exposure to anisomycin resulted in rapid and strong activation of p46/54^{JNK} and p38^{MAPK}, with a delayed low level dual-phosphorylation of mitogen/extracellular protein kinase (p42/44^{MAPK}), low density lipoprotein (LDL) receptor induction depends solely on the mild activation of p42/44^{MAPK} signaling cascade in HepG2 cells. Unlike hepatocyte growth factor (HGF) which caused LDL receptor induction via rapid, strong, and Ras-dependent p42/44^{MAPK} activation, anisomycin-induced p42/44^{MAPK} activity and increased LDL receptor expression in a Ras-independent manner. Finally, we examined the role of the p42/44^{MAPK} signaling cascade in LDL receptor induction by activating this kinase independently of anisomycin or HGF. By using estrogen-dependent human Raf-1 protein kinase in transient transfection assays, we show that the exclusive activation of the Raf-1/MEK-1/p42/44^{MAPK} signaling cascade with antiestrogen ICI 182,780 caused induction of LDL receptor expression to the same level as observed with either HGF or anisomycin. Consistent with the role of p42/44^{MAPK}, induction was strongly inhibited by pretreatment with the MEK-1/2 inhibitor PD98059. Our observation that anisomycin can use p42/44^{MAPK} signaling cascade is a departure from established thinking, and the results presented shows that activation of the p42/44^{MAPK} alone is sufficient to fully induce LDL receptor transcription.—Dhawan, P., A. Bell, A. Kumar, C. Golden, and K. D. Mehta. **Critical role of p42/44^{MAPK} activation in anisomycin and hepatocyte growth factor-induced LDL receptor expression: activation of Raf-1/MEK-1/p42/44^{MAPK} cascade alone is sufficient to induce LDL receptor expression.** *J. Lipid Res.* **1999.** 40: **1911–1919.**

Supplementary key words anisomycin • hepatocyte growth factor • mitogen-activated protein kinase • LDL receptor induction

Mitogen-activated protein kinases (MAPKs) are proline-directed serine–threonine kinases that have important functions as mediators of cellular responses to a variety of extracellular stimuli (reviewed in references 1–4). The MAPKs comprise a family of kinases, the mitogen/extracellular protein kinase (p42/44^{MAPK}), the c-Jun NH_2 terminal kinases (p46/54^{JNK}), and the p38^{MAPK}. Whereas p42/44^{MAPK} are characteristically associated with cell proliferation and protection from apoptosis, p46/54^{JNK} and p38^{MAPK} can promote apoptosis in many systems. Members of the p46/54^{JNK} and p38^{MAPK} subfamilies are strongly activated in response to stress stimuli such as UV radiation, heat shock, major proinflammatory cytokines, and hyperosmolarity. The most potent protein synthesis inhibitor, anisomycin, has been shown to elicit highly specific and strong activation of stress-related p46/54^{JNK} and p38^{MAPK} (5). Due to reported selectivity in its action, there has been a recent resurgence in the use of anisomycin, particularly as an agent that selectively activates these stress-related MAPKs (6–14). The MAPKs are tightly regulated by multicomponent signal transduction cascades consisting of small GTP binding proteins (Ras, Cdc42, and Rac), MAPK kinase kinases (Raf and MEKK), and MAPK kinases (MEKs, MAPKKs, and SEK) (reviewed in references 1, 15). Several downstream targets for MAPKs have also been characterized (3). For example, the p42/44^{MAPK} and one downstream target, the protein kinase RSK, translocates to the nucleus on activation and indirect evidence suggests that in the nucleus, these protein kinases

Abbreviations: MAPK, mitogen-activated protein kinase; p42/44^{MAPK}, mitogen/extracellular protein kinase; p46/54^{JNK}, c-jun NH_2 terminal kinases; HGF, hepatocyte growth factor; MEK, MAPK/ERK kinase; MEKK, MAPK kinase kinase; IL-1β, interleukin-1β; FCS, fetal calf serum; TPA, 12-O-tetradecanoylphorbol-13-acetate; C.A., constitutively active; D.N., dominant-negative; LDL, low density lipoprotein; SS, squalene synthase; ΔRaf-1:ER, conditionally active form of Raf kinase.

[1] Present address: Molecular and Cellular Endocrinology Branch, NIDDK, National Institutes of Health, Bethesda, MD 20892.

[2] To whom correspondence should be addressed (e-mail mehtakamald@exchange.uams.edu).

Downloaded from www.jlr.org by guest, on June 14, 2012

may phosphorylate and regulate the activities of several transcription factors, including Jun, Fos, Elk-1, cyclic AMP responsive element binding protein, and serum response factor (reviewed in reference 4). Similarly, $p38^{MAPK}$ and $p46/54^{JNK}$ also regulate transcription factors like ATF2, Elk-1, and Jun (4).

Anisomycin strongly induces transcription of several immediate early (IE) genes, including c-fos, fos B, c-jun, jun B, and jun D, and the activation of the stress-related kinase has been correlated with its ability to induce their expression (5, 16, 17). Anisomycin has also been shown to induce low density lipoprotein (LDL) receptor transcription in Jurkat cells (18), as it does for the normally labile IE genes (17). Treatment of human fibroblasts with other protein synthesis inhibitors has been shown to induce LDL receptor expression at the transcriptional level (19). The induction of LDL receptor expression in response to protein synthesis inhibitors has been proposed to be a secondary effect of translational arrest that results in the disappearance of a labile repressor (18, 19). Although the existence of such a factor has been postulated for years (20, 21), this labile repressor has not yet been biochemically characterized in eukaryotic cells.

In view of the documented ability of anisomycin to specifically activate $p46/54^{JNK}$ and $p38^{MAPK}$, we set out to investigate the role of these MAPKs in regulating LDL receptor expression. Here we report that anisomycin, unlike puromycin and cycloheximide, specifically induces LDL receptor expression over a wide range of concentrations in HepG2 cells. Although anisomycin strongly activates $p46/54^{JNK}$ and $p38^{MAPK}$, LDL receptor induction actually depends on the mild activation of $p42/44^{MAPK}$ cascade. We also show that not only is the $p42/44^{MAPK}$ cascade required for anisomycin-induced LDL receptor transcription, but activation of this signaling cascade alone is sufficient for induction of LDL receptor transcription. These results highlight the role of $p42/44^{MAPK}$ cascade as an obligatory step in anisomycin signaling and underscore the importance of performing specific controls when assessing roles of the stress-related MAPKs by using anisomycin.

MATERIALS AND METHODS

Materials

Anisomycin, cycloheximide, puromycin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calphostin C, PD98059, and SB202190 were purchased from Calbiochem (San Diego, CA). PD98059 from Research Biochemicals Inc. (Natick, MA) was also used in some experiments. ICI 182,780 was purchased from Tocris (Ballwin, MO). BZA-5B, a specific inhibitor of Ras farnesylation, was a kind gift from Genentech Inc. (South San Francisco, CA). Phospho-specific antibodies to the activated forms of $p42/44^{MAPK}$ (Thr 202/Tyr 204), $p46/54^{JNK}$ (Thr 183/Tyr 188), $p38^{MAPK}$ (Thr 180/Tyr 182), and MAPK/ERK kinase kinase, MEK-1/2 (Ser 217/221) were purchased from New England Biolabs (Beverly, MA). Antibodies to $p42/44^{MAPK}$, MAPK phosphatase-1 (MKP-1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). TRIzol and all tissue culture supplies were from Life Technologies Inc. (Gaithersburg, MD). Zeta probe blotting membrane and the protein assay reagent were purchased from Bio-Rad (Hercules, CA). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from DuPont (Boston, MA) and the enhanced chemiluminescence (ECL) detection kit was obtained from Amersham International (Arlington Heights, IL). Interleukin-1β (IL-1β) and hepatocyte growth factor (HGF) were purchased from R & D Systems (Minneapolis, MN). The chemiluminescent reporter gene assay system for the detection of luciferase was purchased from TROPIX, Inc. (Bedford, MA).

Cell culture

HepG2 cells were maintained as monolayer cultures in a humidified 5% CO_2 atmosphere at 37°C in Eagle's minimum essential medium (EMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Life Technologies Inc.), 2 mm l-glutamine, 20 units/ml penicillin, and 20 μg/ml streptomycin sulfate.

[^{35}S]methionine labeling of HepG2 cells

Cells were grown in a 24-well plate, starved for 16 h in EMEM supplemented with 0.5% fetal calf serum, and washed twice with PBS and once with methionine-free medium. After incubation in this medium at 37°C for 10 min, the medium was replaced by 1 ml of medium containing [^{35}S]methionine (15 μCi/ml) and either anisomycin, cycloheximide, or puromycin at the indicated concentrations. Cells were incubated for 1 h at 37°C, washed twice with EMEM, and then lysed in 200 μl of whole cell lysis buffer (50 mm Tris-HCl [pH 7.5], 100 mm NaCl, 5 mm $MgCl_2$, 1% Triton X-100, 0.1% deoxycholate, protease inhibitor cocktail). Debris were pelleted for 10 min at 10,000 *g*, and 100 μl of the supernatant was precipitated with trichloroacetic acid and collected onto glass filters (Whatman). The filters were washed with 10 ml of 5% trichloroacetic acid followed by 3 ml of 100% ethanol, and then counted.

Immunoblot analysis

Proteins were fractionated by SDS-PAGE with a 10% acrylamide separation gel, and the separated proteins were transferred to nitrocellulose and processed as described previously (22–24). Briefly, membranes were incubated in 20 mm Tris-HCl, pH 7.6, 137 mm NaCl, 0.2% v/v Tween-20 (Tris/NaCl/Tween-20) with 5% w/v non-fat dried milk for 1 h, washed in Tris/NaCl/Tween-20 ($3 \times$ 5 min), and incubated for 1 h with primary antibody in Tris/NaCl/Tween-20 containing 1% milk at room temperature for non-phospho-specific antibodies and overnight at 4°C for phospho-specific antibodies. The following dilutions were used for individual antibodies against different proteins: $p42/44^{MAPK}$ (1:1600); phospho-$p42/44^{MAPK}$ (1:1000); phospho-$p46/54^{JNK}$ (1:1000); phospho-$p38^{MAPK}$ (1:1600); phospho-MEK-1/2 (1:1000); MKP-1 (1:1500). After further washing in Tris/NaCl/Tween-20, membranes were incubated for 1 h with horseradish peroxidase-linked anti-IgG secondary antibody (Bio-Rad, diluted 1:5000), and immunoreactive proteins were detected by ECL as described by the supplier. Quantitative analyses of protein levels were performed by densitometric scanning of the autoradiograms and are representative of 3 or more independent experiments.

Northern analysis

Total RNA was isolated using TRIzol, and Northern blotting was done essentially as described earlier (22–24). Briefly, 20 μg total cellular RNA was fractionated on 1% formaldehyde agarose gel and transferred to Zeta Probe membrane by capillary blotting. RNA blots were hybridized with LDL receptor and squalene synthase-specific single-stranded M13 probes labeled with [α-^{32}P]dCTP. Hybridized filters were washed and exposed to Kodak X-ray film. The relative intensities of specific bands were determined densitometrically within the linear range of the film

Downloaded from www.jlr.org by guest, on June 14, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

on a model 300A laser densitometer (Molecular Dyanamics, CA) with Image Quant software. LDL receptor mRNA was normalized to squalene synthase (SS) (included in this study) or to β-actin (data not shown) mRNA level, and data for each point were plotted as the percentage of LDL receptor mRNA as compared to controls.

Expression vectors and reporter constructs

The human LDL receptor promoter construct used for luciferase assays contained the human LDL receptor promoter from residues −273 kb to +35 cloned upstream of the luciferase gene as described previously (25). Construction of the estradiol-regulated forms of oncogenic Raf-1 (ΔRaf-1:ER) was described earlier (26, 27), and contained the kinase domain of oncogenic Raf-1 fused to the hormone binding domain of the human estrogen receptor. These constructs were provided by Dr. Martin McMahon, Cancer Center, University of California. The dominant-negative (D.N.) and constitutively active (C.A.) mutants of ΔRaf-1:ER contained substitution of tyrosines (Y340 and Y341) with phenylalanine (FF) and aspartic acid (DD), respectively. The expression vectors for MEK1, C.A. MEK1 mutant (MKK1-SS/DD), and D.N. MEK1 (MKK1-S222A) were obtained from Dr. Jacques Pouysse'gur, Centre de Biochimie, CNRS, Nice Cedex, France. Dominant-negative Ras and MKP-1 were obtained from Dr. Jeffrey Pessin, Dept. of Physiology, University of Iowa.

Transient transfection and luciferase assay

HepG2 cells were transfected by the Lipofectamine method as described previously (25). Briefly, HepG2 cells were seeded at a density of 1×10^6 cells per 6-well plates and co-transfected with human LDL receptor promoter-luciferase reporter plasmid (0.5 μg) and 0.25 μg of relevant expression vector or the corresponding empty vector. After transfection, cells were washed three times and maintained in 0.5% FCS with or without IL-1β (5 ng/ml), HGF (10 ng/ml), anisomycin (50 ng/ml), or ICI 182,780 (1 μm) for 8–12 h. Luciferase assays were performed as described before [25].

RESULTS

Anisomycin induces LDL receptor expression in HepG2 cells

We examined the effects of three protein synthesis inhibitors, that are structurally and mechanistically different in the manner in which they inhibit protein synthesis (5), on LDL receptor expression in HepG2 cells. Northern blot analysis of HepG2 cells treated with different concentrations of various inhibitors is shown in **Fig. 1**. At concentrations that completely blocked protein synthesis (**Fig. 2**), puromycin (150 μg/ml) and cycloheximide (5 μg/ml) did not significantly induce LDL receptor transcript, whereas anisomycin strongly induced at all concentrations tested (0.01–5 μg/ml) in medium containing either 0.5% serum (Fig. 1) or 10% serum (results not shown). Anisomycin treatments led to a significant increase in LDL receptor mRNA at concentrations which are insufficient to block protein synthesis. The maximal level of LDL receptor mRNA was reached at a concentration of 50 ng/ml (Fig. 1) which still allowed translation to proceed at 60–70% of its efficiency in the absence of the drug (Fig. 2). Anisomycin was unable to induce expression of another sterol-responsive SS gene (Fig. 1). Induction of LDL

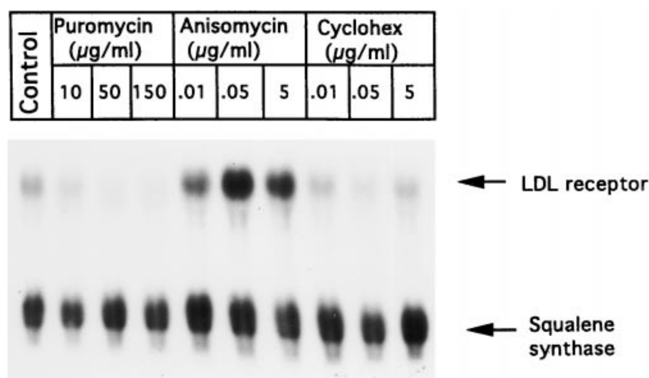


Fig. 1. LDL receptor levels in HepG2 cells treated with puromycin, anisomycin, or cycloheximide at varying concentrations. HepG2 cells (1×10^6/25 cm^2 flask) were plated on day 0 in EMEM supplemented with 10% fetal bovine serum. On day 2, cells were refed with fresh media. On day 4, media containing 0.5% serum and the given concentration of the indicated inhibitor were added to the cells. Total RNA was subjected to Northern blot analysis, and the filter was hybridized with ^{32}P-labeled LDL receptor, SS, and actin cDNA probes. LDL receptor mRNA levels were normalized to actin mRNA levels. The experiment was repeated two times with similar results. Cyclohex, cycloheximide.

receptor expression, even at concentrations of anisomycin below that which inhibits protein synthesis, and the lack of the effect of cycloheximide and puromycin suggest that the inhibition of protein synthesis per se is not sufficient for

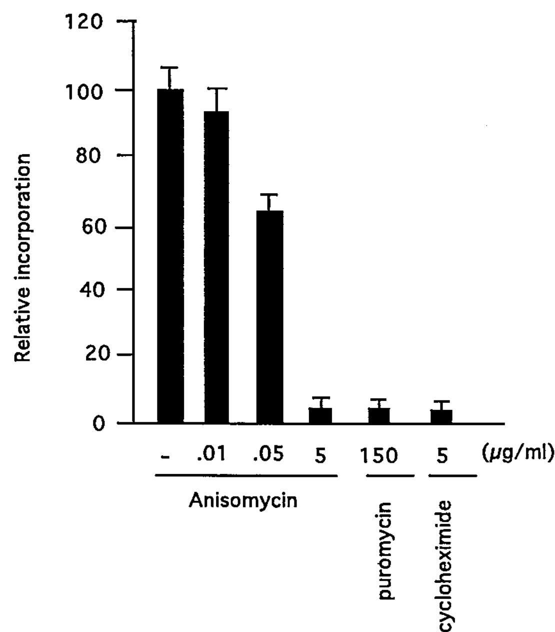


Fig. 2. Protein synthesis levels in HepG2 cells in response to different protein synthesis inhibitors. To determine the inhibitory effects of various concentrations of protein synthesis inhibitors, HepG2 cells were treated for 60 min without (0) or with the indicated concentrations of protein synthesis inhibitors. Incorporation of [^{35}S]methionine was determined in quadriplicate, and the average results were normalized to control values. The results are presented as means ± standard errors and represent at least three individual experiments.

Downloaded from www.jlr.org by guest, on June 14, 2012

ASBMB JOURNAL OF LIPID RESEARCH

anisomycin-induced LDL receptor expression in HepG2 cells. These results clearly rule out the involvement of a continuously synthesized labile protein in significantly repressing LDL receptor gene expression in HepG2 cells.

Anisomycin activates all the three MAPKs in HepG2 cells

In view of numerous recent reports showing that anisomycin acts as a signaling agonist at sub-inhibitory concentrations due to potent and specific activation of p46/54^{JNK} and p38^{MAPK} (6–14), we investigated the activation of three MAPKs by anisomycin in HepG2 cells. We first evaluated the temporal characteristics of MAPKs activation in response to 50 ng/ml of anisomycin in HepG2 cells by using antibodies specific for phosphorylated (activated) forms of the p42/44^{MAPK}, p46/54^{JNK}, p38^{MAPK}, and MEK-1/2. As shown in **Fig. 3A**, anisomycin treatment resulted in rapid and strong activation of the p46/54^{JNK} and p38^{MAPK} with a slight activation of the p42/44^{MAPK}. The magnitude and time course of the activation between p42/44^{MAPK} and p46/54^{JNK} or p38^{MAPK} are different; both p46/54^{JNK} and p38^{MAPK} showed peak activities 30 min post-stimulation, whereas p42/44^{MAPK} was most active at approximately 4 h. The activity of p42/44^{MAPK} was sustained for at least up to 8 h. The increase in activity of p42/44^{MAPK} does not result from an increase in expression of p42/44^{MAPK} protein, as determined by Western blotting with phosphorylation-independent p42/44^{MAPK} antibody (Fig. 3A).

Anisomycin treatment also induced MEK-1/2 phosphorylation (Fig. 3B) with kinetics similar to p42/44^{MAPK} activation, suggesting that anisomycin acts upstream of the p42/44^{MAPK} phosphorylation, at least at the level of MEK-1/2 activation. At the same time, anisomycin did not cause any significant change in the expression of MKP-1 at those time points where maximum activation of MEK-1/2 or MAPKs was observed (Fig. 3B), indicating that the increase in p42/44^{MAPK} activity is not due to a decrease in MKP-1 expression. The above results are consistent with the notion that activation of p42/44^{MAPK} is mediated by MEK-1/2 activation.

Anisomycin-induced LDL receptor expression is mediated through p42/44^{MAPK}

The activation of three MAPKs raised the question of which pathway is required for anisomycin-induced LDL receptor expression. To determine the ability of specific inhibitors of MEK and p38^{MAPK} to affect anisomycin-induced LDL receptor expression, HepG2 cells were treated with varying concentrations of PD98059 (a specific MEK inhibitor) (22–24, 28) or SB202190 (a specific p38^{MAPK} inhibitor) (24, 29, 30). Interestingly, induction was completely prevented by PD98059, but not by SB202190 (**Fig. 4**),

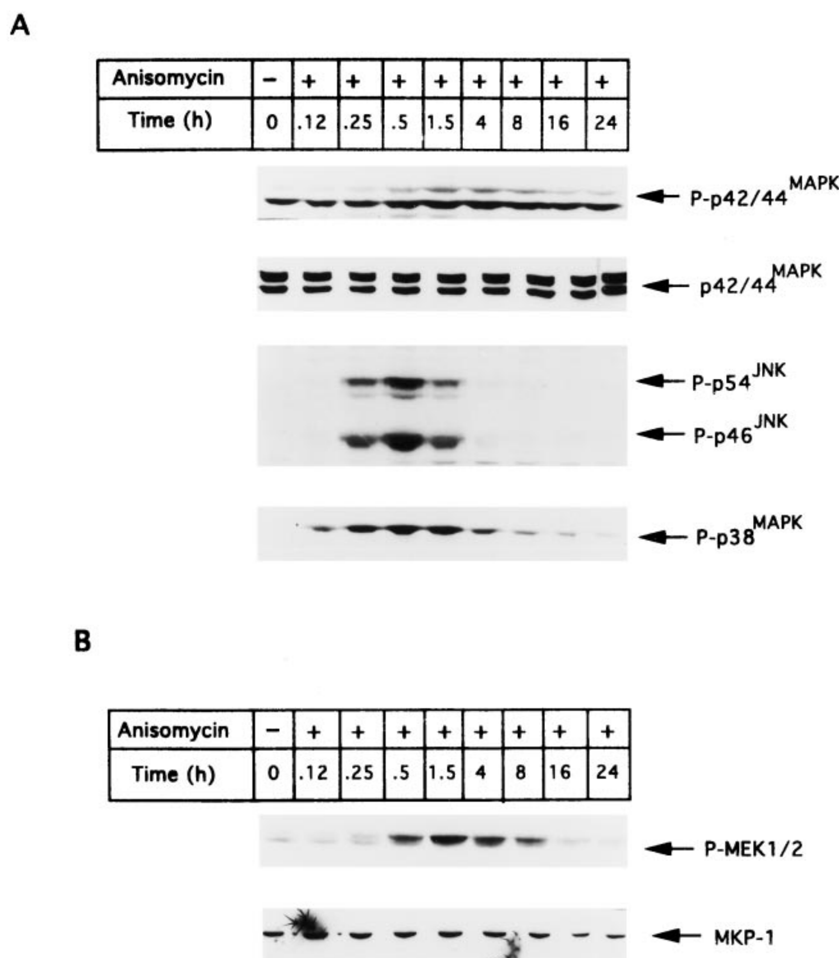
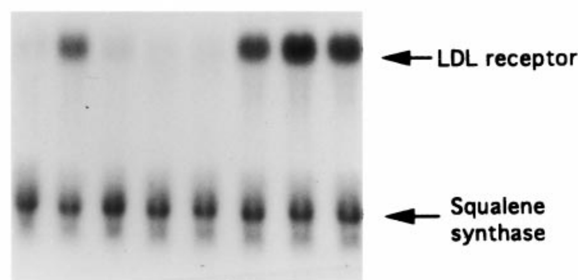


Fig. 3. Kinetics of activation of p42/44^{MAPK}, p46/54^{JNK}, p38^{MAPK}, and MEK-1/2 in HepG2 cells treated with anisomycin. (A) HepG2 cells were grown as described above, and treated with 50 ng/ml of anisomycin for various time intervals in media containing 0.5% serum. After indicated times, cells were washed with phosphate-buffered saline and lysed with 1 × SDS sample buffer. Equal amounts of cell lysates were analyzed by Western blotting using the indicated phospho-specific antibodies. Phosphorylation-independent p42/44^{MAPK} antibody was used to measure expression levels of p42/44^{MAPK} protein. (B) Effects of anisomycin on MEK-1/2 phosphorylation and MKP-1 expression level in HepG2 cells. The cell lysates prepared above were probed with phospho-specific anti-MEK-1/2 or phosphorylation-independent MKP1 antibody. Results shown are indicative of three separate experiments.

Downloaded from www.jlr.org by guest, on June 14, 2012

PD 98059 (μM)	-	-	5	20	50	-	-	-
SB 202190 (μM)	-	-	-	-	-	.5	2.5	5
Anisomycin	-	+	+	+	+	+	+	+



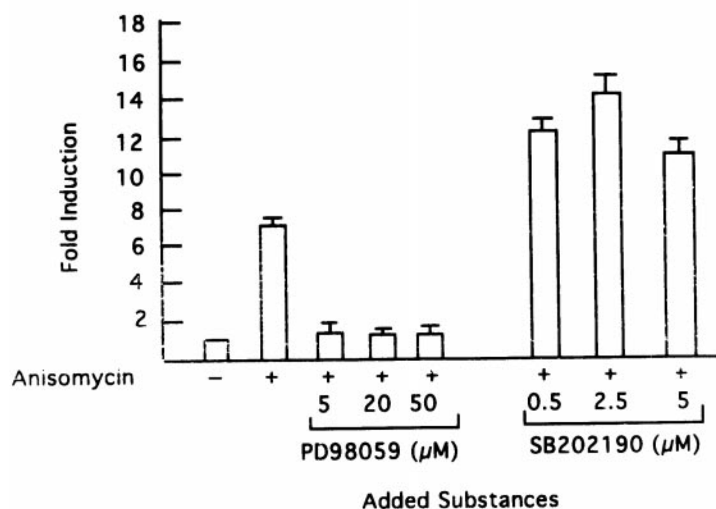


Fig. 4. Effects of PD98059 and SB202190 inhibitors on anisomycin-induced LDL receptor expression in HepG2 cells. Cells pretreated for 30 min with the indicated concentrations of inhibitor were either untreated or treated with anisomycin (50 ng/ml) for 4 h in medium containing 0.5% serum. Total RNA was subjected to Northern blot analysis. Autoradiographs were quantified densitometrically within the linear range of the film on a model 300A laser densitometer (Molecular Dyanamics, CA) and Image Quant software. A representative autoradiogram is shown above, and the results of the densitometric analysis of LDL receptor mRNA levels normalized to actin mRNA (not shown) or to SS are shown below (fold induction over untreated cells (zero time point)).

thereby providing evidence for the crucial involvement of p42/44^{MAPK} activation in anisomycin-induced LDL receptor expression. The role of the p42/44^{MAPK} in anisomycin-induced signaling pathway was surprising in view of only a slight activation of this enzyme, an enzyme characteristically associated with cell proliferation and protection from apoptosis. In contrast, inhibition of p38^{MAPK} signaling pathway by SB202190 resulted in the super-induction of LDL receptor expression in a dose-dependent manner (Fig. 4) without affecting the expression of SS gene. It is likely that this effect is due to cross-talk between the p38^{MAPK} and the p42/44^{MAPK} signaling pathways (24).

Comparison of the effects of BZA-5B, a selective Ras farnesyltransferase inhibitor, on LDL receptor induction and p42/44^{MAPK} activation by anisomycin, IL-1β, and HGF

To study the role of farnesylated Ras in anisomycin-induced LDL receptor expression, we used an inhibitor of Ras farnesyltransferase, the benzodiazepine peptidomimetic BZA-5B (31). BZA-5B has been shown to block attachment of a farnesyl moiety to some Ras isoforms, among other proteins, thereby effectively suppressing Ras biological functions and downstream signaling. In order to deplete cells of farnesylated Ras proteins, cells were pretreated with 50 μm BZA-5B for 3–4 days and then stimulated with either anisomycin, IL-1β, or HGF. HGF was included as a positive control because stimulation of receptor tyrosine kinases usually results in the activation of the Raf-1/MEK/p42/44^{MAPK} through Ras in many cell types (32). As expected, HGF caused rapid and strong activation of p42/44^{MAPK} through MEK-1/2 (**Fig. 5A**), and PD98059 treatment prevented HGF-induced p42/44^{MAPK} activation and an increase in LDL receptor expression (Fig. 5B) demonstrating that the LDL receptor induction by HGF is mainly mediated by the p42/44^{MAPK} (**Fig. 6**). IL-1β was also included for comparative study because we showed previously that this agent induces LDL receptor expression through p42/44^{MAPK} activation (23). Treatment with BZA-5B completely suppressed HGF-induced p42/44^{MAPK} activation and LDL receptor induction (**Fig. 7A** and **7B**). This is perhaps expected as functional Ras is required for transduction of the mitogenic signals from activated receptor tyrosine kinases to p42/44^{MAPK}. On the other hand, IL-1β and anisomycin-induced p42/44^{MAPK} activation and induction of LDL receptor expression were not blocked by BZA-5B treatments (Fig. 7A and B). Similarly, BZA-5B treatments did not significantly reduce p46/54^{JNK} activation by anisomycin (Fig. 7B). From these results, we conclude that unlike HGF, anisomycin may activate p42/44^{MAPK} and

Downloaded from www.jlr.org by guest, on June 14, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

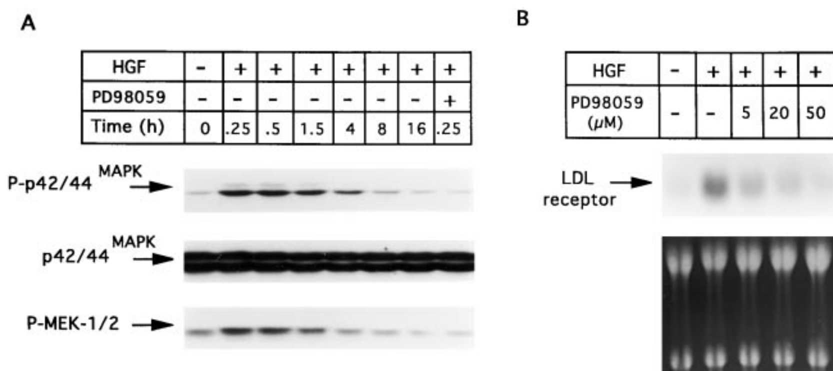


Fig. 5. HGF-induced LDL receptor expression is mediated through p42/44^{MAPK} in HepG2 cells. (A) HepG2 cells were treated with 10 ng/ml HGF in media containing 0.5% serum. Cell extracts were made at the indicated times, and equal amounts of cell extracts were separated by SDS-PAGE and then transferred onto nitrocellulose. Phosphorylation levels of p42/44^{MAPK} and MEK-1/2, and expression levels of p42/44^{MAPK} were determined as described above. In the last lane, cell extracts from HepG2 cells pretreated with 20 μm PD98059 for 30 min were subjected to HGF (10 ng/ml) treatment (20 min). (B) Inhibition of HGF-induced LDL receptor expression by PD98059. HepG2 cells were either untreated or treated with HGF (10 ng/ml) for 4 h either in the absence or presence of indicated concentrations of PD98059 added 30 min prior to 10 ng/ml HGF addition. Ethidium bromide staining of RNA gel before blotting onto a nitrocellulose is also shown to demonstrate equal loading of RNA in all lanes. Results shown are indicative of three separate experiments.

induce LDL receptor expression through Ras-independent pathways in HepG2 cells.

Activation of the p42/44^{MAPK} cascade alone is sufficient to induce LDL receptor expression in HepG2 cells

The above results in conjunction with our earlier findings (22–24) have revealed a critical role for p42/44^{MAPK} activation during induction of LDL receptor expression by a variety of extracellular stimuli. In view of these observations, we wished to examine the contribution of the activation of p42/44^{MAPK} cascade alone on LDL receptor expression in HepG2 cells. We used previously characterized conditionally active Raf-1 expression constructs (ΔRaf-1:ER) in which the kinase domain of oncogenic Raf-1 is fused to the hormone-binding domain of the human estrogen receptor. Estradiol or antiestrogen ICI 182,780 treatments of cells transfected with this construct resulted in a rapid and near-complete activation of the MEK and p42/44^{MAPK} in different cell types, including HepG2 (26, 27, 33). By using transient transfection assays, we determined whether activation of the Raf-1/MEK/p42/44^{MAPK} cascade by ΔRaf-1:ER fusion protein mimics anisomycin or HGF with respect to LDL receptor induction. We cotransfected HepG2 cells with human LDL receptor reporter plasmid A and a vector encoding either C.A. or D.N. form of ΔRaf-1:ER hybrid protein. After 24 h, transfected cells were treated with ICI 182,780 for 15 h at 1 μm, a concentration of hormone that is sufficient to fully activate both ΔRaf:ER fusion proteins (26, 27). A large increase in luciferase reporter gene expression was observed in an ICI-dependent manner in cells transfected with a C.A. ΔRaf-1:ER construct when compared to luciferase expression in cells transfected with either a control vector or with a D.N. ΔRaf-1:ER construct (Fig. 7). Treatment with PD98059 blocked ICI-dependent induction, further confirming that activation of the Raf-1/MEK-1/p42/44^{MAPK} cascade is indeed responsible for increased LDL receptor promoter activity. Similar increase in LDL receptor promoter activity was observed when a constitutively activated form of MEK-1 (MEK-1-SS/DD) was coexpressed in HepG2 cells (Fig. 7). This up-regulation resulted from higher p42/44^{MAPK} activity, as co-expression of MKP-1, a dual specificity phosphatase shown to inactivate p42/44^{MAPK} or PD98059 treatment, totally abolished an increase in LDL receptor promoter-luciferase expression (Fig. 7). Identical results were observed in at least four independent transfection experiments. Taken together, these results demonstrate that activation of the Raf-1/MEK/p42/44^{MAPK} cascade alone initiates positive regulatory signals responsible for LDL receptor expression.

DISCUSSION

Anisomycin can possibly induce LDL receptor expression through different mechanisms. By virtue of its activity as a stimulator of signal transduction cascades, it can cause direct transcriptional, activation. Alternatively, due to its ability to inhibit translation, it might cause the loss of a labile transcriptional repressor or ribonucleases, thereby inducing transcription in an indirect manner or cause mRNA stabilization, respectively. From the studies presented in this paper, we conclude that anisomycin-induced LDL receptor expression cannot be the consequence of translational arrest, first, because neither puromycin nor cycloheximide produced these effects, and second, because induction was observed even at anisomycin concentrations much below that required for efficient inhibition of protein synthesis. Anisomycin led to significant accumulation of LDL receptor mRNA at concentrations that are insufficient to block protein synthesis and the maximal level of LDL receptor mRNA was reached at a concentration of 50 ng/ml which still allowed translation to proceed at about 60–70% of its efficiency in the absence of drugs.

Downloaded from www.jlr.org by guest, on June 14, 2012

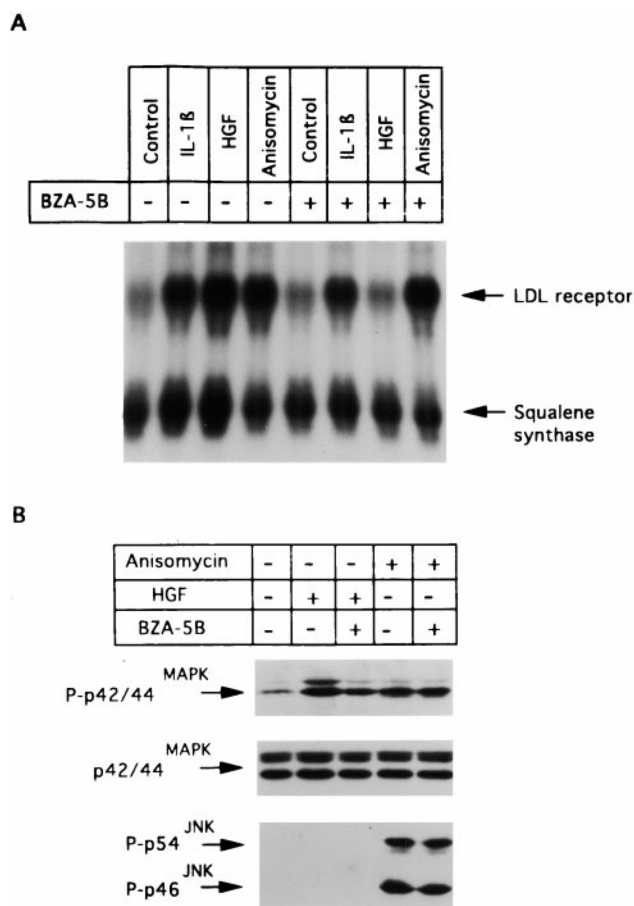


Fig. 6. Effects of BZA-5B on anisomycin-, IL-1β-, and HGF-induced LDL receptor expression and p42/44^{MAPK} activation. (A) HepG2 cells were seeded on day 0. On day 1, 50 μm BZA-5B was added to the cells for 3 days. On day 4, fresh media containing 0.5% serum and 50 μm BZA-5B along with anisomycin (50 ng/ml), IL-1β (5 ng/ml), or HGF (10 ng/ml) were added for 4 h. RNA was isolated and Northern blotting was performed as described above using the LDL receptor and SS cDNA probes. Equal loading of RNA in each lane was confirmed by staining of the RNA gel before blotting onto nitrocellulose. (B) HepG2 cells pretreated with BZA-5B (50 μm) for 5 days were treated with HGF (10 ng/ml) for 15 min, or with anisomycin (50 ng/ml) for 1.5 h in media supplemented with 0.5% serum. Total cell extracts were subjected to Western blotting with phospho-specific p42/44^{MAPK}, phosphorylation-independent p42/44^{MAPK}, or phospho-specific p46/54^{JNK} antibody.

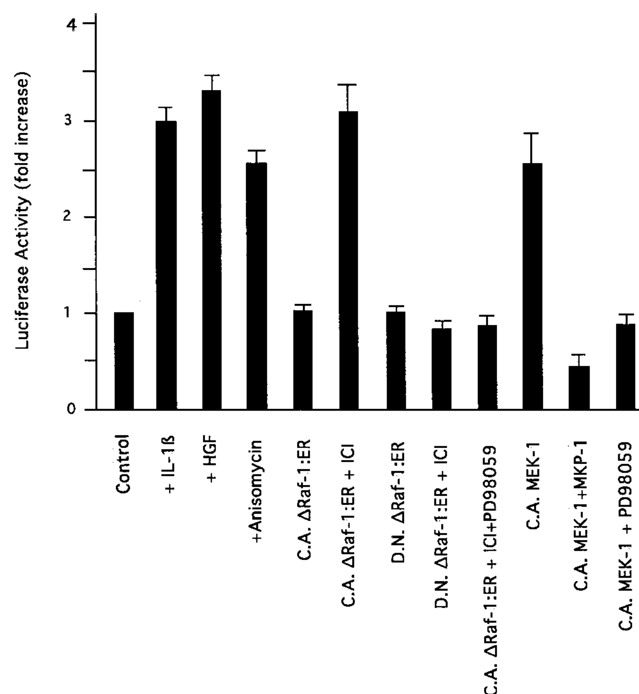


Fig. 7. Effects of dominant negative and constitutively active regulatory enzymes in the Raf-1/MAPK pathway on LDL receptor expression. HepG2 cells were cotransfected with a human LDL receptor-luciferase reporter plasmid (1 μg/plate) with or without the indicated constructs (500 ng each) in the presence or absence of ICI 182,780 as shown. Where indicated, the following were added at the concentration indicated for 8–12 h: IL-1β (5 ng/ml), HGF (10 ng/ml), anisomycin (100 ng/ml), ICI 182,780 (1 μm). After treatment, cells were harvested, and luciferase activity was determined and normalized to the protein content of each extract. The fold increase in luciferase activity on treatment with IL-1β, HGF, or anisomycin was calculated relative to the basal expression level of the untreated cells. For cotransfection experiments, luciferase activity expressed by cells transfected with ΔRaf-1:ER plasmids in the absence of ICI 182,780 was given an arbitrary value of 1 and was corrected for empty vector effects. The results are presented means ± standard errors (error bars) and represent four individual experiments.

These results strongly suggest that anisomycin induces LDL receptor expression by a mechanism independent of protein synthesis.

Results presented herein clearly show that not only is the p42/44^{MAPK} required for LDL receptor transcription, but its activation alone is sufficient for induction of LDL receptor expression. By transfecting estrogen-dependent human Raf-1 protein kinase (ΔRaf-1:ER), we demonstrate that exclusive activation of the Raf-1/MEK-1/p42/44^{MAPK} cascade with ICI 182,780 induces LDL receptor expression to the same levels as observed with either HGF or anisomycin. Furthermore, consistent with the role of this kinase cascade, expression of a constitutively active MEK-1 produced an increase in LDL receptor transcription in the absence of any other growth signal, which was approximately equivalent to the expression level measured in cells stimulated with anisomycin or HGF. Inhibition of Raf- and anisomycin-induced LDL receptor expression by PD98059 further supports the conclusion that the positive signal is mediated through the p42/44^{MAPK} for the induction of LDL receptor expression in HepG2 cells. The mechanism by which p42/44MAPK activation results in LDL receptor induction is not known and is under investigation. Our observation that anisomycin-induced LDL receptor expression at a concentration that inhibited protein synthesis suggests that the transcription factors mediating the induction of LDL receptor gene expression must have been present before the onset of stimulation with anisomycin. By this criterion, LDL receptor can be classified as an IE gene, not requiring de novo synthesis of the transcriptional stimulators. Establishment of a link between the p42/44^{MAPK} and anisomycin-induced LDL receptor expression could potentially explain super-induction of IE

Downloaded from www.jlr.org by guest, on June 14, 2012
ASBMB JOURNAL OF LIPID RESEARCH

genes by anisomycin via direct promoter activation without the existence of a labile repressor. For example, by prolonging the normally transient p42/44^{MAPK} activation in response to cytokines, hormones, and TPA, anisomycin may make an additional contribution to the prolonged transcriptional activation of IE genes under superinducing conditions. In addition, involvement of the p42/44^{MAPK} in anisomycin signaling can also account for the identical phosphorylation of non-histone mobility-group protein HMG-14 on treatment with either anisomycin or mitogen (34).

The role of p42/44^{MAPK} in anisomycin signaling cascade in HepG2 cells is somewhat unexpected, due to numerous reports suggesting that anisomycin-induced changes in gene expression are mediated through the p46/54^{JNK} and/or p38^{MAPK} in other cell types. Although the profile of anisomycin-activated stress-related MAPKs in HepG2 cells is similar to that reported earlier for various non-hepatic cells, activation of the p42/44^{MAPK} by anisomycin may be due to significant heterogeneity between cell types. Such differences have been observed earlier between neuronal and non-neuronal cell types. For example, cAMP has been reported to inhibit p42/44^{MAPK} activation in fibroblasts, and in contrast, activates this kinase in PC-12 cells (35). It appears that differential expression of B-Raf in PC-12 cells contributes to cell type-specific differences in the regulation of p42/44^{MAPK} signaling cascade. Similar differences have been observed in the upstream regulation of stress-related MAPK cascades between the PC-12 and KB cell types (36). Thus, it is likely that the differences observed in the present study may reflect differences in the relative regulation of the p42/44^{MAPK} and the stress-related MAPK pathways between hepatic and non-hepatic cell types. Furthermore, activation of all three MAPKs by anisomycin should not be surprising because the specificities of activating stimuli for the three subfamilies of MAPKs are not absolute; for example, inflammatory cytokines IL-1β and tumor necrosis factor-α are known to activate p42/44^{MAPK} in many cell types, including HepG2 cells (23), and some growth factors can produce a weak activation of the p46/54^{JNK} and p38^{MAPK} (37). Based on the above results, we propose that one should exercise a great deal of caution in extrapolating results using anisomycin.

To elucidate whether Ras is required for anisomycin-induced activation of p42/44^{MAPK} and LDL receptor induction, we performed experiments using BZA-5B, an inhibitor of Ras farnesyltransferase. We found that although HGF-induced p42/44^{MAPK} activation and LDL receptor induction was completely suppressed by treatment with BZA-5B, anisomycin-induced activation was not attenuated by this manipulation, suggesting that the anisomycin-induces LDL receptor expression in a Ras-independent manner. Therefore, the upstream signaling pathways resulting in the activation of the MEK are different for HGF and anisomycin. This observation is consistent with our results showing that anisomycin and HGF induce p42/44^{MAPK} activity with different kinetics. HGF-induced p42/44^{MAPK} activation is rapid and strong, whereas anisomycin induce activation is slow and mild. The delay in anisomycin-induced p42/44^{MAPK} is most likely due to the late appearance of an intermediate signaling component through de novo synthesis, post-translational modification, or localization. The involvement of Ras-independent pathway(s) in the activation of p42/44^{MAPK} and p46/54^{JNK} in HepG2 cells is interesting in view of a recent report that shows anisomycin-induced activation of the p46/54^{JNK} cascade requires Ras in neuronal PC-12 cells (36). This type of difference further supports the existence of cell type-specific differences in the regulation of MAPKs.

In conclusion, results presented in this paper emphasize a general point of significance, namely, although one specific signaling pathway may appear to dominate a response, another mildly activated pathway can be critical, and thus should not be overlooked or ignored. The requirement of p42/44^{MAPK} cascade for TPA (22), cytokines (23), HGF, and anisomycin (this paper)-induced LDL receptor expression suggests that a diverse array of extracellular signals utilize p42/44^{MAPK} signaling cascade to induce LDL receptor expression in HepG2 cells. Promoter dissection studies in progress should lead us to the identification of the crucial DNA sequence motives involved in LDL receptor induction in response to the above agents. The transcription factor(s) interacting with such sequence motives are expected to be the critical targets of the p42/44^{MAPK} signal transduction pathway. From the above studies, it appears that the LDL receptor gene is a very useful tool to investigate the regulation of gene expression by the p42/44^{MAPK} signaling pathway. Moreover, differential activation of the p42/44^{MAPK} cascade may alter cellular events that contribute to differences in expression and responses of LDL receptor gene in a cell-specific manner.

We warmly thank all investigators who supplied the plasmids indicated under Experimental Procedures. We thank R.P. Singh and G. Kapoor for their help with some of the experiments. We also thank Caroline Simmons for critically reading the manuscript. We acknowledge Genentech Inc. for their generous gift of BZA-5B. This work was supported by research grants from the National Institutes of Health (HL-51592-04), Established Investigator Grant from the American Heart Association (9940163N), and UAMS Hornick Endowment award to K. D. M.

Manuscript received 11 February 1999 and in revised form 3 June 1999.

REFERENCES

1. Marshall, C. J. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell.* **80:** 179–185.
2. Seger, R., and E. G. Krebs. 1995. The MAPK signaling cascade. *FASEB J.* **9:** 726–735.
3. Treisman, R. 1996. Regulation of transcription by MAP kinase cascades. *Curr. Opin. Cell Biol.* **8:** 205–215.
4. Robinson, M. J., and M. H. Cobb. 1997. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* **9:** 180–186.
5. Cano, E., and L. C. Mahadevan. 1995. Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci.* **20:** 117–122.
6. Swantek, J. L., M. H. Cobb, and T. D. Geppert. 1997. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha

Downloaded from www.jlr.org by guest, on June 14, 2012

ASBMB JOURNAL OF LIPID RESEARCH

(TNF-α) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. *Mol. Cell. Biol.* **17:** 6274–6282.
7. Weinbrenner, C., G. S. Liu, M. V. Cohen, and J. M. Downey. 1997. Phosphorylation of tyrosine 182 of p38 mitogen-activated protein kinase correlates with the protection of preconditioning in the rabbit heart. *J. Mol. & Cell. Cardiol.* **29:** 2383–2391.
8. Kuroki, D. W., A. Minden, I. Sanchez, and E. V. Wattenberg. 1997. Regulation of a c-jun amino-terminal kinase/stress-activated protein kinase cascade by a sodium-dependent signal transduction pathway. *J. Biol. Chem.* **272:** 23905–23911.
9. Morley, S. J., and L. McKendrick. 1997. Involvement of stress-activated protein kinase and p38/RK mitogen-activated protein kinase signaling pathways in the enhanced phosphorylation of initiation factor 4E in NIH 3T3 cells. *J. Biol. Chem.* **272:** 17887–17893.
10. Moxham, C. M., A. Tabrizchi, R. J. Davis, and C. C. Malbon. 1996. Jun N-terminal kinase mediates activation of skeletal muscle glycogen synthase by insulin in vivo. *J. Biol. Chem.* **271:** 30765–30773.
11. Park, J., I. Kim, Y. Oh, K. Lee, P. L. Han, and E. J. Choi. 1997. Activation of c-jun N-terminal kinase antagonizes an anti-apoptotic action of Bcl-2. *J. Biol. Chem.* **272:** 16725–16728.
12. Bogoyevitch, M. A., A. J. Ketterman, and P. H. Sugden. 1995. Cellular stresses differentially activate c-Jun N-terminal protein kinases and extracellular signal-regulated protein kinases in cultured ventricular myocytes. *J. Biol. Chem.* **270:** 29710–29717.
13. Shu, J., M. Hitomi, and D. Stacey. 1996. Activation of JNK/SAPK pathway is not directly inhibitory for cell cycle progression in NIH3T3 cells. *Oncogene.* **13:** 2421–2430.
14. Nahas, N., T. F. Molski, G. A. Fernandez, and R. I. Shaafi. 1996. Tyrosine phosphorylation and activation of a new mitogen-activated protein (MAP)-kinase cascade in human neutrophils stimulated with various agonists. *Biochem. J.* **318:** 247–253.
15. Waskiewicz, A. J., and J. A. Cooper. 1995. Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeasts. *Curr. Opin. Cell Biol.* **7:** 798–805.
16. Bravo, R. 1990. Growth factor-responsive genes in fibroblasts. *Cell Growth Differ.* **1:** 305–309.
17. Hazzalin, C. A., R. L. Panse, E. Cano, and L. C. Mahadevan. 1998. Anisomycin selectively desensitizes signaling components involved in stress kinase activation and fos and jun induction. *Mol. Cell. Biol.* **18:** 1844–1854.
18. Makar, R. S., P. E. Lipsky, and J. A. Cuthbert. 1994. Non-sterol regulation of low density lipoprotein receptor gene expression in T cells. *J. Lipid Res.* **35:** 1888–1895.
19. Mazzone, T. K., K. Basheeruddin, and H. Duncan. 1989. Inhibitors of translation induce low density lipoprotein receptor gene expression in human skin fibroblasts. *J. Biol. Chem.* **264:** 15529–15539.
20. Greenberg, M. E., A. L. Hermanowski, and E. B. Ziff. 1986. Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin gene transcription. *Mol. Cell. Biol.* **6:** 1050–1057.
21. Subramaniam, M., L. J. Schmidt, C. E. Crutchfield, and M. J. Getz. 1989. Negative regulation of serum-responsive enhancer elements. *Nature.* **340:** 64–66.
22. Kumar, A., T. C. Chambers, B. A. Cloud-Heflin, and K. D. Mehta. 1997. Phorbol ester-induced low density lipoprotein receptor gene expression in HepG2 cells involves protein kinase C-mediated p42/44MAP kinase activation. *J. Lipid Res.* **38:** 4220–4228.
23. Kumar, A., A. Middleton, T. C. Chambers, and K. D. Mehta. 1998. Differential roles of extracellular signal-regulated kinase-1/2 and $p38^{MAPK}$ in interleukin-1β and tumor necrosis factor-α-induced low density lipoprotein expression in HepG2 cells. *J. Biol. Chem.* **273:** 15742–15748.
24. Singh, R. P., P. Dhawan, C. Golden, G. S. Kapoor, and K. D. Mehta. 1999. One-way cross-talk between p38MAPK and p42/44MAPK: Inhibition of p38MAPK a-isoform induces LDL receptor expression through activation of the p42/44MAPK cascade. *J. Biol. Chem.* **274:** 19593–19600.
25. Mehta, K. D., R. Chang, J. Underwood, J. Wise, and A. Kumar. 1996. Identification of a novel *cis*-acting element participating in maximal induction of the human low density lipoprotein receptor gene transcription in response to low cellular cholesterol levels. *J. Biol. Chem.* **271:** 33616–33622.
26. Bosch, E., H. Cherwinski, D. Peterson, and M. McMahon. 1997. Mutations of critical amino acids affect the biological and biochemical properties of oncogenic A-Raf and Raf-1. *Oncogene.* **15:** 1021–1033.
27. Woods, D., D. Parry, H. Cherwinski, E. Bosch, E. Lees, and M. McMartin. 1997. Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. *Mol. Cell. Biol.* **17:** 5598–5611.
28. Pang, L., T. Sawada, S. J. Decker, and A. R. Saltiel. 1995. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J. Biol. Chem.* **270:** 13585–13588.
29. Lee, J. C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, J. R. Heys, S. W. Landvatter, J. E. Strickler, M. M. McLaughlin, I. R. Siemens, S. M. Fisher, G. P. Livi, J. R. White, J. L. Adams, and P. R. Young. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature.* **372:** 739–746.
30. Cuenda, A., J. Rouse, Y. N. Doza, R. Meier, P. Cohen, T. F. Gallagher, P. R. Young, and J. C. Lee. 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364:** 229–233.
31. James, G. L., J. L. Goldstein, M. S. Brown, T. E. Rawson, T. C. Somers, R. S. McDowell, C. W. Crowley, B. K. Lucas, A. D. Levinson, and J. J. Marsters. 1993. Benzodiazepine peptidomimetics: potent inhibitors of Ras farnesylation inanimal cells. *Science.* **260:** 1937–1942.
32. Adachi, T., S. Nakashima, S. Saji, T. Nakamura, and Y. Nozawa. 1996. Mitogen-activated protein kinase activation in hepatocyte growth factor-stimulated rat hepatocytes: involvement of protein tyrosine kinase and protein kinase C. *Hepatology.* **23:** 1244–1253.
33. Dhawan, P., M. McMartin, and K. D. Mehta. 1999. LDL receptor expression is regulated positively by the p42/44MAPK pathway in hepatic cells. Experimental Biology '99, Washington DC, April 17–21, 1999.
34. Barratt, M. J., C. A. Hazzalin, N. Zhelev, and L. C. Mahadevan. 1994. A mitogen- and anisomycin-stimulated kinase phosphorylates HMG-14 in its basic amino-terminal domain in vivo and on isolated mononucleosomes. *EMBO J.* **13:** 4524–4535.
35. Erhardt, P., J. Troppmair, U. R. Rapp, and G. M. Cooper. 1995. Differential regulation of Raf-1 and B-Raf and Ras-dependent activation of mitogen-activated protein kinase by cyclic AMP in PC12 cells. *Mol. Cell. Biol.* **15:** 5524–5530.
36. Kawasaki, H., T. Moriguchi, S. Matsuda, H. Zen Li, S. Makamura, S. Simohama, J. Kimura, Y. Gotoh, and E. Nishida. 1996. Ras-dependent and Ras-independent activation pathways for the stress-activated-protein kinase cascade. *Eur. J. Biochem.* **241:** 315–321.
37. Auer, K. L., J. Contessa, S. Brenz-Verca, L. Pirola, S. Rusconi, G. Cooper, A. Abo, M. P. Wymann, R. J. Davis, M. Birrer, and P. Dent. 1998. The Ras/Rac1/Cdc42/SEK/JNK/c-Jun cascade is a key pathway by which agonists stimulate DNA synthesis in primary cultures of rat hepatocytes. *Mol. Biol. Cell.* **9:** 561–573.

Downloaded from www.jlr.org by guest, on June 14, 2012