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Ultraviolet-induced Phosphorylation of $p70^{S6K}$ at Thr^{389} and Thr^{421}/Ser^{424} Involves Hydrogen Peroxide and Mammalian Target of Rapamycin but not Akt and Atypical Protein Kinase C[1]

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ABSTRACT

The p70 S6 kinase ($p70^{S6k}$) is a Ser/Thr kinase that plays an important role in cell growth, transformation, and the transition of the cell cycle in mammalian cells. Because UV radiation has been reported to induce activation of $p70^{S6k}$, which is believed to play some role in the carcinogenic effects of sun exposure, the present study investigated the signaling pathways involved in this activation induced by UV radiation in mouse epidermal JB6 Cl41 cells. Exposure of cells to UV radiation led to marked increases in $p70^{S6k}$ activity and phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. UV radiation also generated reactive oxygen species as measured by electron spin resonance and by H_2O_2 and O_2^- fluorescence staining assays in JB6 Cl 41 cells. The scavenging of UV-generated H_2O_2 by *N*-acety-L-cyteine (a general antioxidant) or catalase (a specific H_2O_2 scavenger) inhibited $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}, whereas pretreatment of cells with sodium formate (an ·OH radical scavenger) or superoxide dismutase (an O_2^- radical scavenger) did not show any inhibitory effects. Importantly, UV-induced increases in $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} were dramatically inhibited by pretreatment of cells with rapamycin, LY294002, or PD98059, whereas overexpression of dominant-negative mutants of PKCλ/ι and Akt1 did not inhibit $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. These results demonstrated that H_2O_2, phosphatidylinositol 3-kinase, and mammalian target of rapamycin were important players for UV-induced $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}, whereas Akt and atypical protein kinase C were not involved in this activation. The role of H_2O_2 in $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} was further supported by the findings that treatment of cells with H_2O_2 also caused $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}.

INTRODUCTION

There is abundant evidence from previous studies that excessive UV exposure is a major etiological factor in the development of human skin cancer (1). UV-induced epidermal tumors have been studied extensively in various animal models (2–5). UV radiation is believed to act not only as a tumor initiator at the initiation stage of tumor development because of its direct effects on cellular DNA (4, 5) but also as a tumor promoter by eliciting alternations of gene expressions (4, 5). Although the mechanism behind the tumor-inductive ability of UV radiation is not well understood, it is believed that signals from both the cellular membrane and the nucleus initiated by UV radiation are involved in this process (6–9). One of the pathways originates from the nucleus with direct or indirect DNA damage at the primary signal, followed by a transfer of a signal to the cytoplasm, thereby activating specific signaling pathways leading to activation of transcription factors, which eventually return to the nucleus and induce gene expression (6, 7). The second pathway in the UV response involves the generation of ROS[3] near or within the cellular membrane, which subsequently elicit the protein kinase cascades, and in turn activate transcription factors and lead to gene expression (8, 9). Our previous studies have demonstrated that the pathway whereby UV activates AP-1 activity does require aPKC activation (10, 11). Overexpression of a dominant-negative aPKC mutant impaired AP-1 activation via a suppressing activation of ERKs (12).

$p70^{S6k}$ is a Ser/Thr kinase that is stimulated by a variety of mitogens, such as insulin, 12-*O*-tetradecanoylphorbol-13-acetate, and growth factors (13–15). The activation of $p70^{S6k}$ is attributable to phosphorylation of Ser/Thr residues on multiple sites, such as Thr^{389}, Ser^{424}, and Thr^{421} (16–18). Upon activation, $p70^{S6k}$ phosphorylates the S6 protein of the 40S ribosomal subunit (19). Phosphorylated S6 directs the translational machinery toward increasing the production of translational machinery components, such as ribosomal proteins and elongation factors (20). Thus, $p70^{S6k}$ plays an important role in cell growth, transformation, and transition of cell cycle in mammalian cells. For this reason, signal transduction pathways leading to activation of $p70^{S6k}$ have attracted considerable attention in the last few years (13–20). It is believed that PI3K and its downstream effector, the protein kinase Akt, act as signaling intermediates that link cell surface receptors to $p70^{S6k}$ (19, 20). It has also been reported that the PI3K structurally related enzyme, mTOR (also termed FRAP or RAFT), is also involved in the regulation of phosphorylation of $p70^{S6k}$ (20). It was found recently that hydrogen peroxide could induce activation of $p70^{S6k}$ through a pathway upstream of the rapamycin-sensitive component FRAP/RAFT and wortmannin-sensitive PI3K (21). Very recently, Parrott and Templeton (22) reported that UV radiation also induced activation of $p70^{S6k}$ in kidney 293 cells and CV1 cells. However, the molecular mechanisms and signal transduction pathways by which UV causes increased phosphorylation of $p70^{S6k}$ remain unclear. In the present study, we investigated this issue in mouse epidermal JB6 Cl41 cells and found that UV exposure induces $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} through H_2O_2 generation by UV radiation.

MATERIALS AND METHODS

Plasmids, Reagents, and Antibodies. The Akt mutant plasmid, SRα-Akt-T308A/S473A, has a COOH-terminal influenza virus hemagglutinin epitope tag, which is easy detected in transfected cells using a specific hemagglutinin antigen tag antibody (23). Deferoxamine, NAC, β-$NADT^+$ (NADPH), SOD,

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[3] The abbreviations used are: ROS, reactive oxygen species; aPKC, atypical protein kinase C; PKB, protein kinase B; ERK, extracellular signal-regulated kinase; $p70^{S6k}$, p70 S6 kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; NAC, *N*-acetyl-L-cysteine; SOD, superoxide dismutase; HE, HE, dihydroethidium; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; FBS, fetal bovine serum; ESR, electron spin resonance; LDH, lactate dehydrogenase; NF-κB, nuclear factor-κB; AP-1, activated protein-1; PDK, 3-phosphoinositide-dependent protein kinase.

sodium formate, and HE were purchased from Sigma Chemical Co. (St. Louis, MO); H_2DCFDA was from Molecular Probe (Eugene, OR). FBS and Eagle's MEM were purchased from BioWhittaker (Walkersville, MD). Phospho-specific p70^{S6k} (Thr^{389}) antibody, phospho-specific p70^{S6k} (Thr^{421}/Ser^{424}) antibody, p70^{S6k} antibody phospho-specific Akt (Thr308) antibody, phospho-specific Akt (Ser473) antibody, Akt antibody, phospho-specific ERK antibody, and ERK antibody were purchased from New England Biolabs (Beverly, MA). PD98059, a specific ERK pathway inhibitor, and rapamycin, a p70^{S6k} pathway inhibitor, were purchased from Calbiochem (La Jolla, CA).

Cell Culture and Sources of UV Radiation. The JB6 P$^+$ mouse epidermal cell Cl 41 and its transfectant, Cl 41 DN-PKCλ mass1, were cultured in monolayers at 37°C, 5% CO_2 using MEM containing 5% FBS, 2 mM L-glutamine, and 25 μg of gentamicin/ml as described previously (10, 12, 24, 25). Both UVB and UVC lamps were purchased from UVP, Inc. (Upland, CA). UVC lamps generate 254 nm wavelength UV light, whereas UVB lamps generate >95% of 302-nm wavelength UVB light and some UVC light. The UVB radiation used in this study was filtered with a Kodak Kodacel K6808 filter that eliminates all wavelengths <290 nm as indicated in our previous studies (26).

Cellular Superoxide (O_2^-) and H_2O_2 Staining Assays. HE is a specific O_2^- dye (27, 28), whereas H_2DCFDA has been used frequently to monitor H_2O_2 levels in cells (27, 28). The cells were cultured on coverslips in 100-mm dishes until 90% confluent. The cells were then treated with UVB radiation (4 KJ/m^2). The coverslips were washed three times with PBS, and HE or H_2DCFDA (both dissolved in DMSO and diluted with PBS to final concentrations of 5 μM) was applied to the cells and incubated for another 15–20 min at 37°C. The coverslips were washed twice with PBS and observed under a fluorescence microscope.

ESR Measurements. ESR spin trapping was used to detect short-lived free radical intermediates. This technique involves the addition-type reaction of a short-lived radicals with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be measured by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splittings of the spin adduct are generally characteristic of the trapped radical. ESR measurements were carried out using a Varian E9 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. Cl 41 cells were seeded in 100-mm dishes and cultured until 90% confluent. The cells were washed once with PBS; the PBS plus 400 mM DMPO and 100 μM NADPH were added to each dish. The cells were then exposed to UVB radiation (4 KJ/m^2). The cells were harvested and transferred to a flat cell for ESR measurement as described previously (28, 29).

Transient Transfection. Cl 41 cells were cultured in a 6-well plate until they reached 85–90% confluence. Fifteen μl of LipofectAMINE reagent with 15 μg of Akt-T308A/S473A plasmid DNA were used to transfect cells of each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM. Approximately 24–30 h after the beginning of the transfection, the cells were exposed to either UVB (4 KJ/m^2) or UVC (60 J/m^2) and culture. Cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were used for Western blot.

Western Blot Analysis. JB6 C141 cells (2×10^4) were cultured in each well of 6-well plates to 90% confluent with 5% FBS MEM. Cells were exposed to either UVB (4 KJ/m^2) or UVC (60 J/m^2) and cultured for the time indicated. The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with one of the antibodies at 1:500 dilution. The antibodies used in this study included rabbit phospho-specific p70^{S6k} (Thr^{389}) antibody, phospho-specific p70^{S6k} (Thr^{421}/Ser^{424}) antibody, p70^{S6k} antibody, phospho-specific Akt (Thr^{308}) antibody, phospho-specific Akt (Ser^{473}) antibody, Akt antibody, phospho-specific Erk antibody, and Erk antibody. The protein bands specifically bound with primary antibodies were detected using an antirabbit IgG-AP-linked and ECF Western blotting system according to the manufacturer's recommendations (30).

p70^{S6K} Activity Assay. JB6 C141 cells were cultured in 150-mm dishes to 80% confluent with 5% FBS MEM. Cells were exposed to either UVB or UVC at doses as indicated and cultured for 150 min after exposure. The cells were harvested by washing with ice-cold PBS and lysed on ice for 40 min in 1 ml of cold immunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EGTA] supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF. The lysates were cleared by centrifugation. The protein concentration in the lysates was determined using Bio-Rad protein assay reagent. The proteins (200 μg) in 300 μl of immunoprecipitation assay buffer were incubated with 1.5 μg of anti-p70^{S6k} antibody (Santa Cruz Biotechnology) for 1 h at 4°C. The immunocomplex was incubated with 30 μl of protein A/G-agarose (50% slurry; Santa Cruz Biotechnology) for 1 h at 4°C. The beads were washed two times with 300 μl of cold PBS followed by one wash with assay dilution buffer [20 mM 4-morpholinepropanesulfonic acid (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT]. p70^{S6k} activities were measured by using a S6 assay kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, the beads were resuspended in 20 μl of assay dilution buffer, 10 μl of substrate, 10 μl of inhibitor mixture, and 10 μl of [γ-^{32}P]ATP mixture (75 mM $MgCl_2$, 500 μM ATP, 10 μCi of [γ-^{32}P]ATP]. The reaction mixtures were incubated for 10 min at 30°C and then centrifuged for 1 min. Aliquots (20 μl) of the supernatants were spotted onto a P81 phosphocellulose filter and washed three times for 15 min using 0.75% phosphoric acid, followed by one wash in acetone. The filters were transferred to scintillation vials and counted in a Wallace 1410 liquid scintillation counter (Perkin-Elmer). Mean kinase activity data from three replicated experiments were normalized to results obtained in cells without the treatment of UV light (control). The data were presented as the mean and SE values.

LDH Assay. JB6 C1 41 cells (5×10^4) suspended in 1 ml of 5% FBS MEM were added to each well of a 24-well plate and cultured in monolayers at 37°C, 5% CO_2 incubator. Twelve h later, cells were starved in Eagle's MEM supplemented with 0.1% FBS for 40 h and then exposed to UVB or UVC at different doses as indicated in the same medium. The cells were incubated at 37°C for another 8 h. The supernatant of the cell culture was centrifuged at 1500 rpm for 5 min. The LDH activity in the supernatant was quantified using COBAS MIRA systems according to the protocol provided by the manufacturer.

RESULTS

Induction of p70^{S6k} Activity and Phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} by UV Radiation. It has been reported that UV radiation induced the activation of p70^{S6k} in kidney 293 cells and CV1 cells (22). Because the skin is the major target organ for UV radiation, we exposed mouse epidermal Cl 41 cells to either UVB or UVC radiation. The results showed that both UVB and UVC markedly induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} in mouse epidermal Cl41 cells at all doses used without cytotoxicity (Fig. 1, *A* and *C*). This induction appeared to be dose dependent (Fig. 1*A*). Maximum induction of p70^{S6k} phosphorylation occurred between 4 and 7 h after cells were exposed to UV radiation (Fig. 1*B*). Consistent with p70^{S6k} phosphorylation, UV radiation also led to increases in p70^{S6k} activity (Fig. 1*D*). These results demonstrated that UV radiation is a potent stimulus for induction of p70^{S6k} activation in mouse epidermal cells.

Generation of ROS by UV Radiation. Previous studies indicated that UV-induced signaling involved the ROS generation and that exposure of cells to H_2O_2 resulted in p70^{S6k} activation (21, 31). We proposed that UV-induced p70^{S6k} phosphorylation may be mediated by ROS. To determine ROS generation directly in cultured cells, dye staining and ESR techniques were used. Measurements using HE, a specific fluorescent dye for O_2^-, or H_2DCFDA, a fluorescent dye for H_2O_2, demonstrated that exposure of cells to UVB radiation led to an increase in the generation of both O_2^- and H_2O_2 (Fig. 2). The increases in cellular O_2^- or H_2O_2 could be dramatically scavenged by incubation of cells with SOD or catalase, respectively (Fig. 2). The results from ESR studies showed that cells without UVB radiation or UVB radiation without cells did not generated any detectable amount of free radicals (Fig. 3, *a*

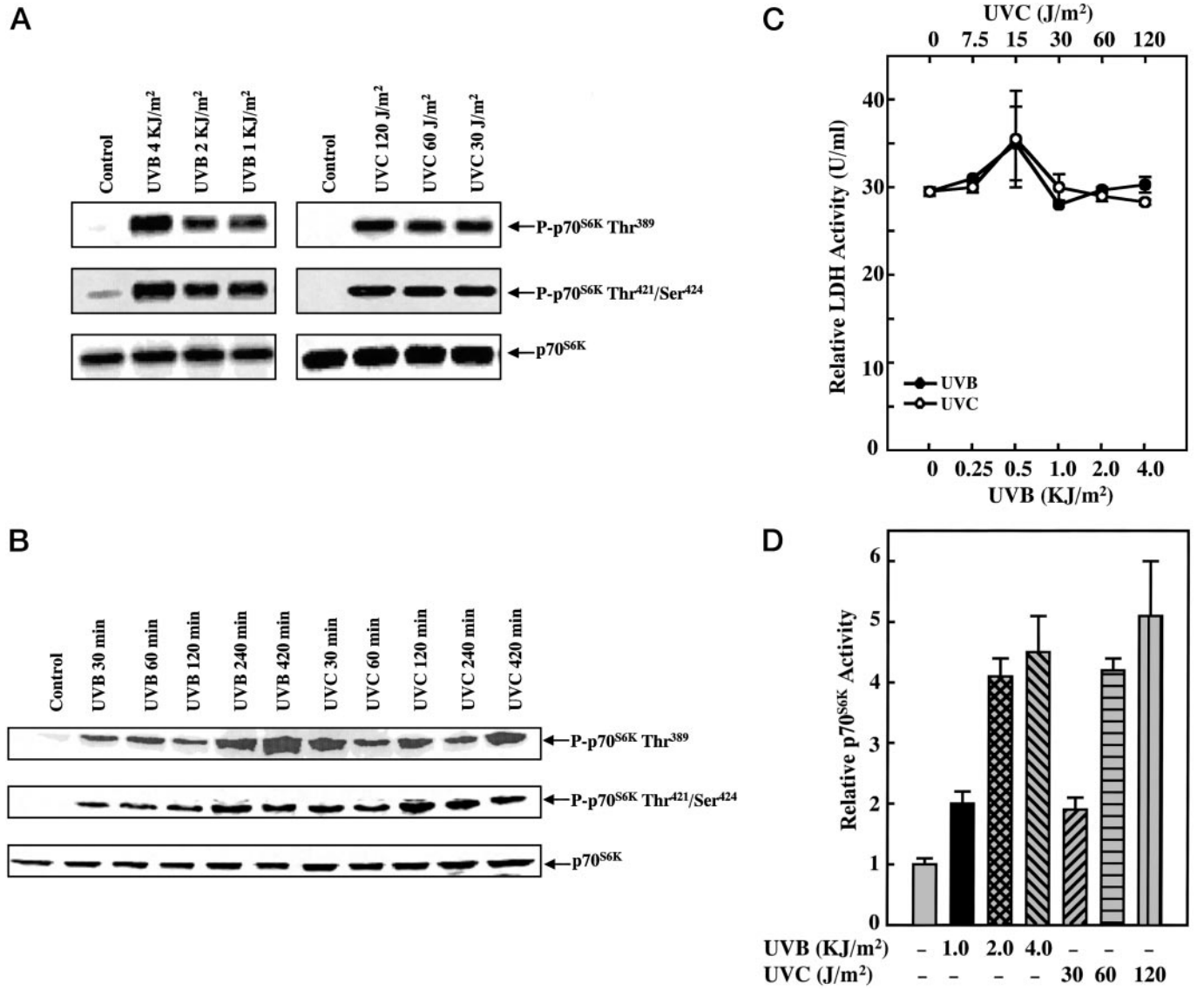

Fig. 1. Induction of p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} by UV radiation. Subconfluent (90%) monolayers of JB6 C1 41 cells in 100-mm dishes were subjected to UV radiation at the doses as indicated and cultured for 4 h (*A*); or either UVB (4 KJ/m^2) or UVC (60J/m^2) and cultured for various times as indicated (*B*). The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with one of the antibodies, including rabbit phospho-specific p70^{S6k} (Thr^{389}) antibody, phospho-specific p70^{S6k} (Thr^{421}/Ser^{424}) antibody, or p70^{S6k} antibody. The p70^{S6k} protein band specific binding with primary antibodies was detected by using an antirabbit IgG-AP-linked and ECF Western blotting system (32). *C*, an LDH assay was used to test the cytotoxicity of UV radiation in Cl41 cells. Briefly, 5×10^4 of C141 suspended in 5% FBS MEM were added to each well of a 24-well plate and cultured. Twelve h later, cells were starved in 0.1% FBS MEM for 40 h and then exposed to UVB or UVC in the same medium. The cells were incubated at 37°C for another 8 h. The supernatant of the cell culture was harvested, and the LDH activity in the supernatant were quantified as described in "Materials and Methods." *D*, JB6 C141 cells were cultured in 150-mm dishes to 80% confluent with 5% FBS MEM. Cells were exposed to UV radiation and harvested at 150 min after exposure. p70^{S6k} activities were measured by using a S6 assay kit according to the manufacturer's instructions. The data were presented as relative p70^{S6k} activity of the means; *bars*, SE.

and *b*). However, exposure of cells to UVB radiation generated a 1:2:2:1 ESR spectrum (Fig. 3*c*) with hyperfine splittings of $a_H = a_N = 14.9$ G, where a_N and a_H denote hyperfine splittings of the nitroxyl nitrogens and α-hydrogen, respectively. On the basis of these splittings and the 1:2:2:1 line shape, the spectrum was assigned to the DMPO/·OH adduct, which is evidence of ·OH radical generation. It was noted that ESR showed high peak of ·OH and was generated at 7 min by UVB radiation, whereas fluorescent dye staining show increases in O_2^- and H_2O_2 were observed at 20 min after cells were exposed to UVB radiation. The reason for this difference is attributable to the two ROS assays used. ESR spin trapping detects short-lived free radical intermediates. This technique involves the addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped. Therefore, the level of ·OH determined by ESR is the transient level of ·OH generation in cells. In contrast, the fluorescent dye staining to measure O_2^- and H_2O_2 is to determine the accumulation of O_2^- and H_2O_2 from the beginning of UV exposure in the cells. Because sensitivity of fluorescent dye staining is not as high as ESR, it takes a longer time to accumulate more fluorescent products to be observed. The results from this study with UVB, together with the results in our previous studies with UVC (32), provide direct and strong evidence that both UVB and UVC radiation are able to induce the generation of a whole spectrum of ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) in mouse epidermal cells, suggesting that ROS generation by UV might be involved in the activation of signal transduction pathways.

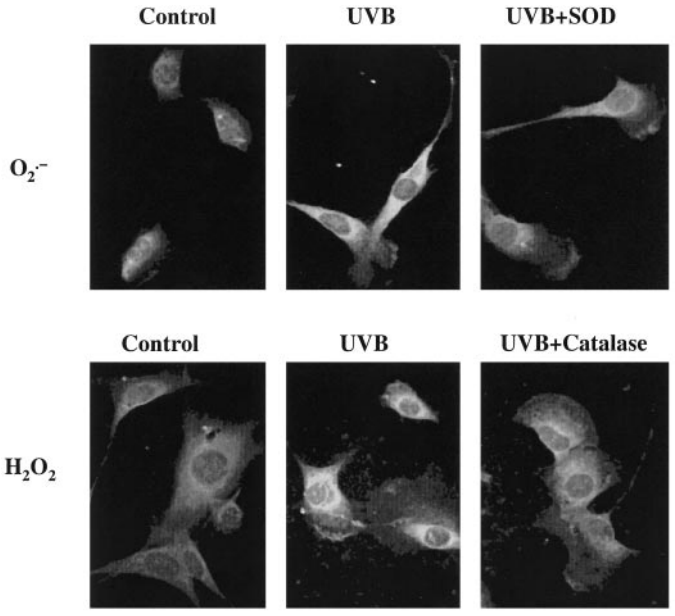

Fig. 2. Determination of O_2^- and H_2O_2 by HE and H_2DCFDA staining. Cl41 cells were cultured on coverslips in 100-mm dishes until 90% confluent. The cells were preincubated in the absence or presence of SOD (250 units/ml) or catalase (5×10^4 units/ml) for 30 min and then exposed to UVB radiation (4 KJ/m^2). The coverslips were washed three times with PBS, and then HE or H_2DCFDA (both dissolved in DMPO and diluted with PBS to final concentrations of 5 μM) was applied to the cells on the coverslips and incubated for another 15–20 min at 37°C. The coverslips were washed twice with PBS and observed using a fluorescence microscope.

Generation of H_2O_2 Is Required for p70^{S6k} Phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} by UV Radiation. To obtain direct evidence for the involvement of ROS in p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} in UV responses, the effects of specific ROS modifiers on UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} were determined. Pretreatment of cells with NAC, a general antioxidant, or catalase, a specific H_2O_2 scavenger, inhibited UV-induced phosphorylation of p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} as compared with UVB and UVC radiation, respectively (Fig. 4). In contrast, treatment of cells with sodium formate, an ·OH radical scavenger, did not inhibit UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} (Fig. 4, *A* and *B*). These inhibitions were further confirmed by dose-response studies (Fig. 4, *C* and *D*). It was noted that there was some difference between UVB and UVC for inhibitory effects by NAC or catalase. It appeared that UVB-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} was more sensitive to NAC and catalase than that induced by UVC. This might be attributable to more ROS generation by UVC than that by UVB. This was supported by our data recently that the ESR spectrum peak generated by UVC is higher than that generated by UVB (Fig. 5). It should be noted that the addition of SOD or NADPH did not substantially increase p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} induced by UV radiation (Fig. 4, *A* and *B*). A reason for this might be that the generation of H_2O_2 by relative high doses of UV radiation is strong enough to induce maximum p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} in Cl 41 cells. The explanation is based on the results that SOD or NADPH did increase p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} induced by low doses of UV radiation (Fig. 6). These data suggest that H_2O_2 generation by UV radiation is involved in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}.

Induction of p70^{S6k} Phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} by H_2O_2. The results from above the studies revealed that UV radiation could generate ROS and that UV-generated H_2O_2 was required for UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} in Cl41 cells. If this is the case, exposure of cells to H_2O_2 should also induce p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. The results showed that treatment of cells with H_2O_2 indeed resulted in increased p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} in a time- and dose-dependent manner (Fig. 7). Because various cellular antioxidants, such as ascorbate, glutathione, and NADPH, and pyruvate, catalase, and glutathione peroxidase will react with H_2O_2 (33–35), the H_2O_2 concentration used in this study was relative high. This may be the reason that low concentration (<50 μM) of H_2O_2 had no induction of p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} (Fig. 7*B*). The results supported the notion that H_2O_2 was the mediator for UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}.

H_2O_2-mediated p70^{S6k} Phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} by UV Radiation Is Through a Rapamycin-sensitive and PI3K-dependent Pathway. The identification of kinases involved in p70^{S6k} phosphorylation is a crucial step in elucidating the signal transduction pathways leading to p70^{S6k} activation. The results from previous reports indicated that mTOR, PI3K, Akt, and aPKC were involved in p70^{S6k} phosphorylation induced by a variety of stimuli (20). To study the role of mTOR and PI3K in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}, we first observed the effects of rapamycin and a PI3K inhibitor, LY294002, on UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. The results showed that pretreatment of cells with either rapamycin or LY294002 resulted in a dramatic inhibition of p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} as compared with UVB and UVC, respectively (Fig. 8). It was noted that UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} was very sensitive to rapamycin, because rapamycin could completely block this phosphorylation at concentration as low as 5 μM (Fig. 8). The strong inhibitory effects of rapamycin were not attributable to the cytotoxicity because it did not affect UV-induced ERKs phosphorylation, even at 40 μM (Fig. 8). One should also note that the extent of inhibition of p70^{S6k} phosphorylation at Thr^{389} is greater than that at Thr^{421}/Ser^{424} by LY294002 (Fig. 8, *C* and *D*). These results revealed that there might be different PI3K downstream kinases responsible for phosphorylation of the two sites. The above data indicate that both mTOR and PI3K were required for p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}.

Role of the ERKs cascade in UV-induced p70^{S6k} Phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. Because p70^{S6k} and ERKs pathways are associated with cell growth, it is reasonable to ask whether there is any cross-talk between the two pathways. PD98059 is a chemical-specific inhibitor of mitogen-activated protein kinase kinase 1/2, which is a direct upstream kinase specifically responsible for activating ERKs. To test whether there was any cross-talk between the ERK pathways and the p70^{S6k} pathways, we used PD98059 and rapamycin. Pretreatment of cells with PD98059 showed a partial

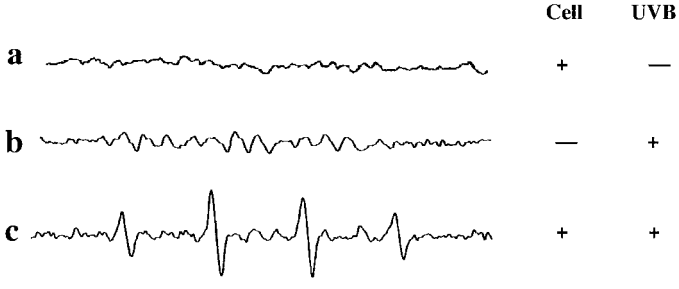

Fig. 3. Measurement of UV-induced ROS generation by ESR. ESR spectra were recorded 7 min after cells were exposed to UVB (4 KJ/m^2) in a 100-mm dish with 90% confluent Cl41 cells, in PBS plus 400 mM DMSO and 100 μM NADPH.

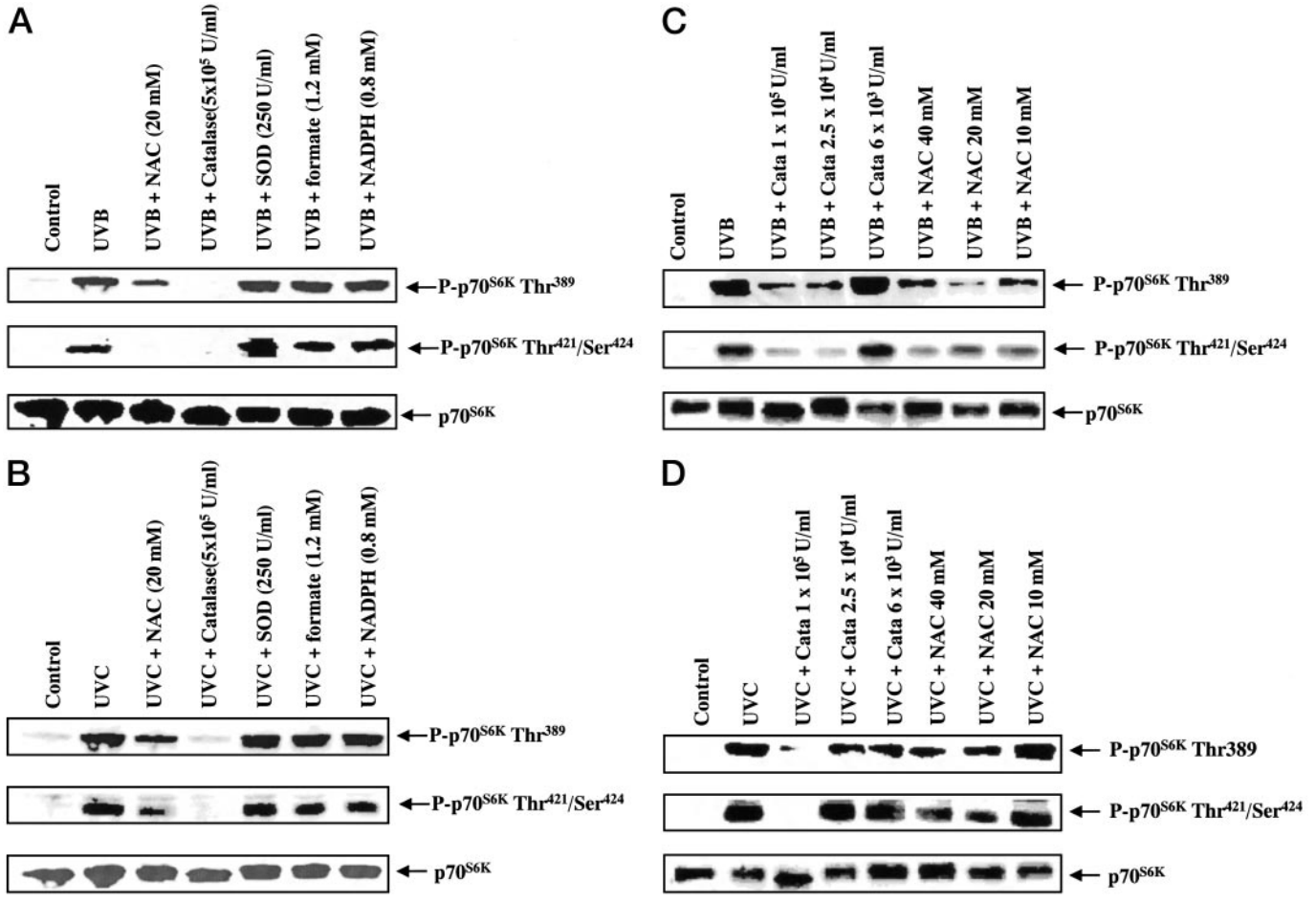

Fig. 4. Effects of ROS modifiers on UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. JB6, C1 41, suspended in 5% FBS MEM, were added to each well of 6-well plates and cultured overnight. The cells were pretreated with different ROS modifiers as indicated for 30 min. The cells were then exposed to UVB (4 KJ/m^2; *A* and *C*) or UVC (60J/m^2; *B* and *D*) and cultured for 120 min. The cells were harvested, and Western blot analysis was carried out as described in Fig. 1. *Cata*, catalase.

inhibitory effect on UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} as compared with UVB and UVC, respectively (Fig. 8, *A* and *B*). However, rapamycin did not affect UV-induced ERKs phosphorylation (Fig. 8, *A* and *B*). These data reveal that ERKs might play some role in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}.

aPKC and Akt Are Not Required for p70^{S6k} Phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} by UV Radiation. To investigate the role of aPKC in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}, we compared the p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} between the stable transfectants of dominant-negative mutant PKCλ (C141 DN-PKCλ mass1) and vector control (Cl41 AP-1 mass1; Refs. 10–12). C141 DN-PKCλ mass1 is a well-characterized stable transfected cell line (10–12), which was established with *Xenopus* dominant-negative mutant PKCλ plasmid, pRcCMVλ^{mut} (36). pRcCMVλ ^{mut} is a kinase-defective mutant of PKCλ, which was shown to block tumor necrosis factor-α-induced NF-κB activation (35). Our previous studies have demonstrated that an overexpression of DN-PKCλ in C141 DN-PKCλ mass1 cells impairs UV-induced AP-1 activity (10, 11). The impairment of UV-induced AP-1 activation by an overexpression of DN-PKCλ was mediated by specific inhibition of UV-induced ERK activation (12). As shown in Fig. 7, there is no difference for UV-induced p70^{S6k} phosphorylations at Thr^{389} and Thr^{421}/Ser^{424} between C141 DN-PKCλ mass1 and Cl41 AP-1 mass1 (Fig. 9, *A–D*). In contrast, UV-induced Akt phosphorylation at Ser^{473} and Thr^{308} was impaired in C141 DN-PKCλ mass1 as compared with Cl41 AP-1 mass1 (Fig. 9, *C* and *D*). These data suggest that aPKC did not play a role in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. To study the role of Akt in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}, we used an Akt mutant, Akt-T308A/S473A, in which these two amino acids were replaced with alanines, and could not be activated by phosphoinositide-dependent kinases, PDK-1 and PDK-2 (36). The results showed that overexpression of the dominant-negative mutant Akt1, Akt-T308A/S473A, blocked UV-induced Akt phosphorylation at Ser^{473} and Thr^{308} as compared with vector control, Cl41 SRα (Fig. 9*E*), whereas it did not show any inhibitory effects on UV-induced

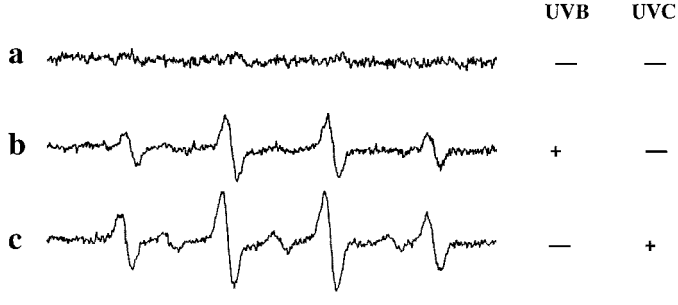

Fig. 5. Comparison of ESR signals generated by UVB and UVC. ESR spectra were recorded 2 min after cells exposed to either UVB (4 KJ/m^2) or UVC (60J/m^2) in a 100-mm dish with 90% confluent Cl41 cells, in PBS plus 400 mM DMPO and 100 μM NADPH.

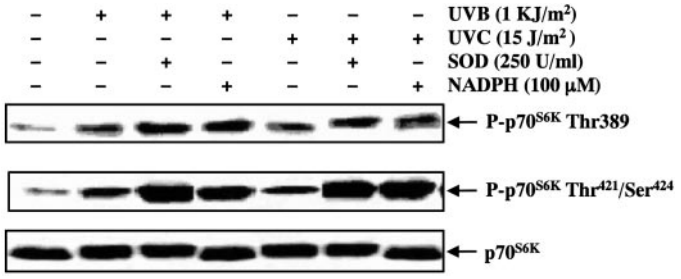

Fig. 6. Effects of SOD and NADPH on low doses of UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. C1 41 cells, suspended in 5% FBS MEM, were added to each well of 6-well plates and cultured overnight. The cells were treated with either SOD (250 units/ml) or NADPH (100 μM). The cells were then exposed to UVB (1 KJ/m^2) or UVC (15 J/m^2) and cultured for 120 min. The cells were harvested, and Western blot analysis was carried out as described in Fig. 1. *P-*, phosphorylated.

p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} (Fig. 9*E*). The result suggests that Akt activation was not involved in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}.

DISCUSSION

p70^{S6k} is activated upon UV exposure. The activation of p70^{S6k} by UV radiation is attributable to phosphorylation of p70^{S6k} at multiple sites, presumably by multiple kinases. However, the signal transduction pathways leading to activation of p70^{S6k} in UV responses are not well understood. The results presented in the present study demonstrate that UV radiation leads to the generation of a whole spectrum of ROS, including $O_2^{\cdot-}$, H_2O_2, and ·OH. Among these ROS, H_2O_2 appears responsible for UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} in mouse epidermal Cl 41 cells. This conclusion is based on the following observations: (*a*) UV radiation lead to generation of H_2O_2; (*b*) catalase, a specific scavenger of H_2O_2, inhibited UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}; (*c*) pretreatment of cells with SOD or sodium formate did not inhibit UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}, revealing that the $O_2^{\cdot-}$ and ·OH radicals are not involved in this process; (*d*) H_2O_2 alone was able to induce p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. Furthermore, we found that mTOR, PI3K, and ERK, but not Akt or aPKC, play a role in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}.

ROS are one of the important determinants in the regulation of cell functional pathways involved in proliferation, apoptosis, and transformation (37–42). Intercellular levels of ROS are influenced by a number of endogenous and exogenous processes and are regulated by several radical scavenger enzymes (40). Exogenous agents that induce ROS generation include chemical and physical carcinogens and various cytokines (28, 40). It is well accepted that extracellular stimuli trigger signals through a cascade of protein-protein interactions (28, 29, 30, 43). It is generally believed that these extracellular stimuli generate and/or require reactive free radicals or derived oxidant species to successfully transmit their signals to the nucleus (41, 42). Therefore, ROS also function as intracellular messengers (40, 41). The cells overexpressing catalase were unable to activate NF-κB in response to tumor necrosis factor-α and okadaic acid (44). The catalase inhibitor aminotriazole restored the NF-κB response (44). In contrast, overexpressing cytosolic SOD, which causes cytosolic hydrogen peroxide accumulation, potentiated the NF-κB response (44). The results presented here demonstrate that increased levels of intracellular H_2O_2 and p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} were detected upon exposure of cells to UV radiation. Pretreatment of cells with catalase prevented the increase in H_2O_2 and resulted in inhibition of p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. These data suggest that UV-generated H_2O_2 plays an essential role in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. It was observed that there is a differential p70^{S6k} phosphorylation between sites of Thr^{389} and Thr^{421}/Ser^{424} after cells were exposed to H_2O_2. The maximum induction of p70^{S6k} phosphorylation at Thr^{389} occurred at 120 min after cells were exposed to H_2O_2, whereas the maximum p70^{S6k} phosphorylation at Thr^{421}/Ser^{424} was observed at 60 min of exposure (Fig. 5*a*), suggesting that there might be differential kinases involved in p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. This hypothesis was supported by the data that phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} shows differential sensitivities to NAC and PI3K inhibitor.

There is evidence indicating that activation of PI3K and subsequent activation of the PKB pathway is involved in the activation of p70^{S6k}. p70^{S6k} is activated by expression of constitutively active PI3K (45, 46), and p70^{S6k} activation is blocked by dominant-negative forms of PI3K or by PI3K inhibitors (46, 47). The role of PKB in the activation of p70^{S6k} is suggested by the findings that expression of constitutively active PKB led to p70^{S6k} activation (48). However, there is some indication of difference between Akt and p70^{S6k} pathways, because no direct p70^{S6k} phosphorylation has yet been demonstrated in cells that express constitutively active PKB (49), and dominant-negative Akt failed to inhibit p70^{S6k} activation (48). The present study shows that mTOR, ERKs, and PI3K play a role in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}, whereas Akt is not required for this process.

The observation that the p70^{S6k} activation is dependent on PI3K, but not Akt, has raised the question of whether aPKC is involved in the pathway leading to p70^{S6k} activation. It has been reported that dominant-negative aPKC antagonized p70^{S6k} activation by epidermal growth factor (50), whereas a myristoylated, constitutively active

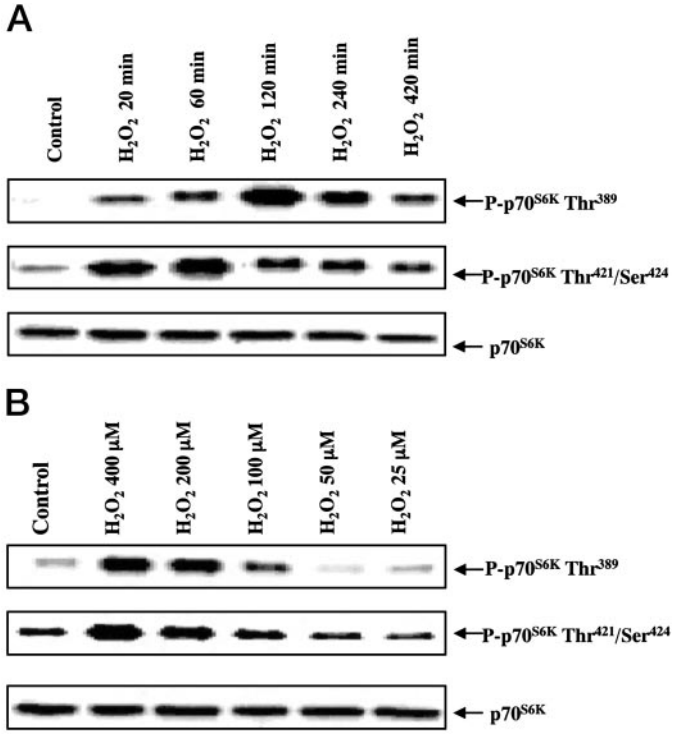

Fig. 7. Induction of p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} by H_2O_2. Subconfluent (90%) monolayers of JB6 C1 41 cells in 100-mm dishes were subjected to 200 μM of H_2O_2 for various times as indicated (*A*) or different doses of H_2O_2 for 120 min (*B*). Cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The Western blot was carried out as described in Fig. 1. *P-*, phosphorylated.

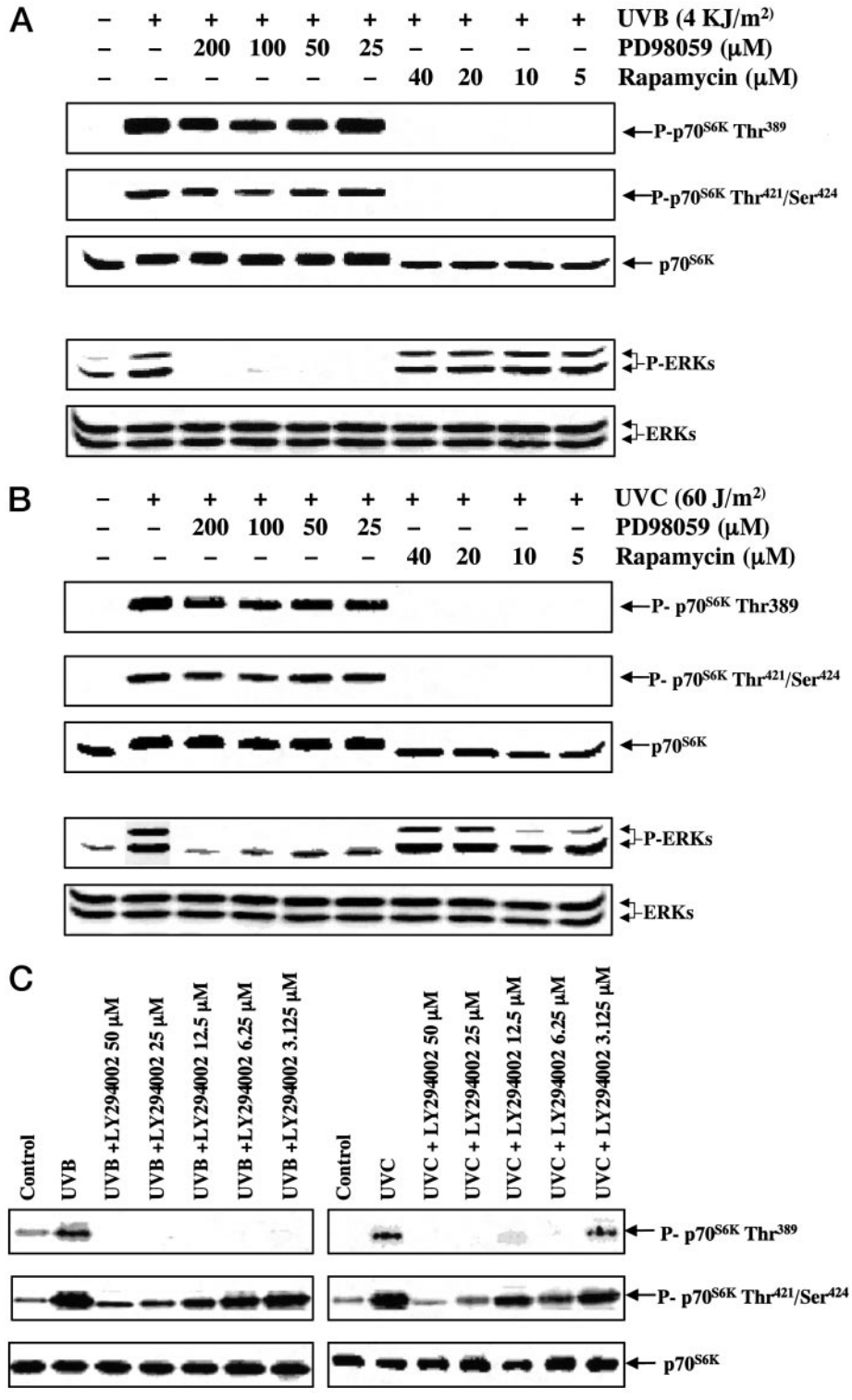

Fig. 8. Effects of rapamycin, LY294002, or PD98059 on UV-mediated p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. C1 41 cells suspended in 5% FBS MEM were added to each well of 6-well plates and cultured overnight. The cells were pretreated with different concentrations of rapamycin or PD98059 (*A* and *B*) or LY294002 as indicated for 30 min (*C* and *D*). The cells were then exposed to UVB (4 KJ/m^2; *A* and *C*) or UVC (60J/m^2; *B* and *D*) as indicated and cultured for 120 min. The cells were harvested, and the Western blot was carried out as described in Fig. 1. *P*-, phosphorylated.

aPKC induced a modest activation of p70^{S6k} (50). Because our previous studies demonstrated that UV radiation caused marked activation of aPKC and this activation was required for UV-induced AP-1 activation in JB6 cells (10, 11), we investigated the role of aPKC in UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. The results show that overexpression of dominant-negative mutant PKCλ does not exhibit any inhibitory effects on UV-induced p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}, whereas it blocks UV-induced Akt phosphorylation and activation of AP-1 and ERKs (10–12). These data suggest that aPKC is not involved in the pathway leading to p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} by UV radiation.

In conclusion, UV radiation generates ROS and induces p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. Among ROS generated by UV radiation, H_2O_2 is the mediator for p70^{S6k} phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. Considering the important role of p70^{S6k} in the regulation of cell growth and the cell cycle, the present study suggests that H_2O_2-mediated p70^{S6k} phosphoryl-

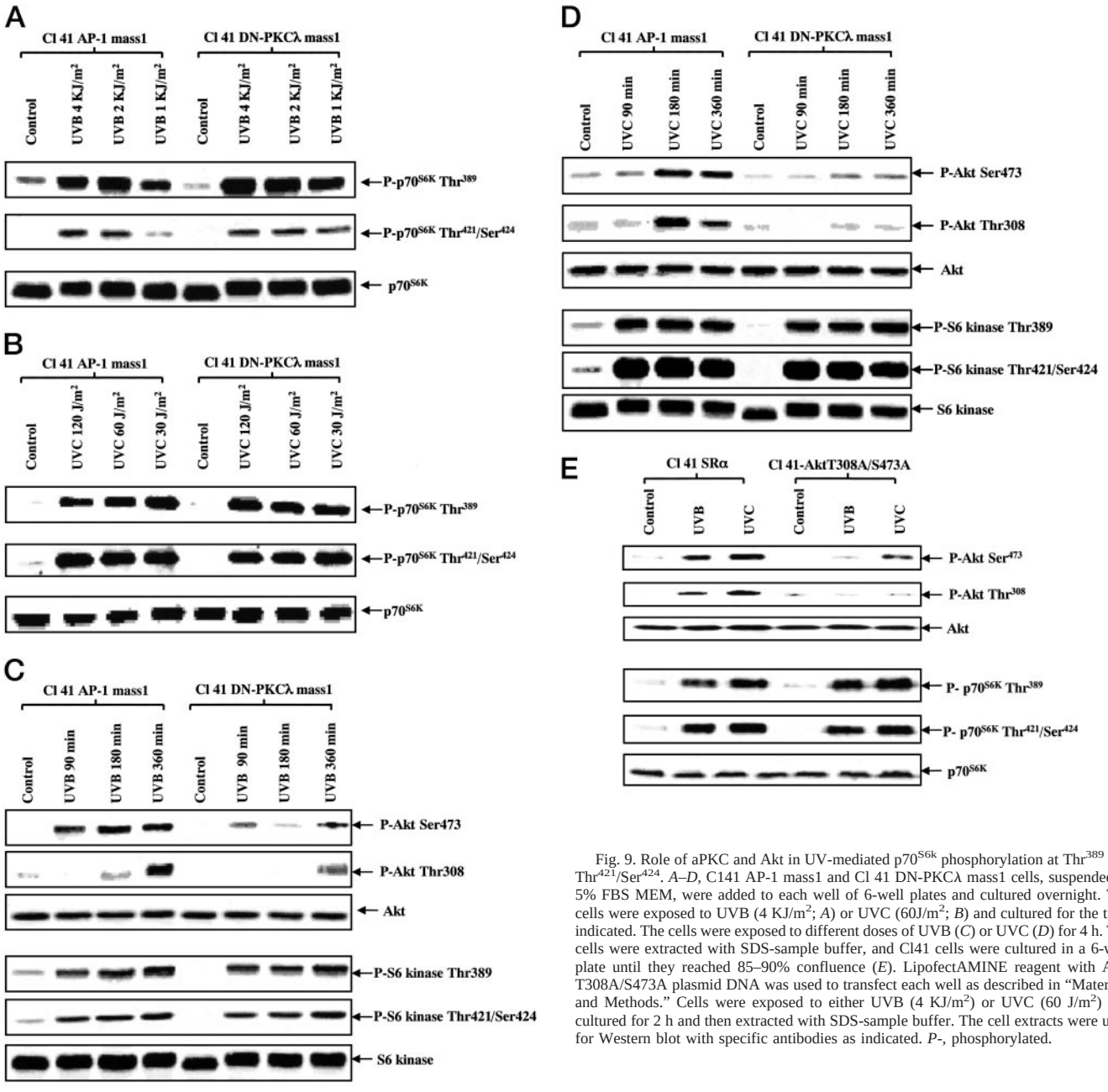

Fig. 9. Role of aPKC and Akt in UV-mediated $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. *A–D*, C141 AP-1 mass1 and Cl 41 DN-PKCλ mass1 cells, suspended in 5% FBS MEM, were added to each well of 6-well plates and cultured overnight. The cells were exposed to UVB (4 KJ/m^2; *A*) or UVC (60J/m^2; *B*) and cultured for the time indicated. The cells were exposed to different doses of UVB (*C*) or UVC (*D*) for 4 h. The cells were extracted with SDS-sample buffer, and Cl41 cells were cultured in a 6-well plate until they reached 85–90% confluence (*E*). LipofectAMINE reagent with Akt-T308A/S473A plasmid DNA was used to transfect each well as described in "Materials and Methods." Cells were exposed to either UVB (4 KJ/m^2) or UVC (60 J/m^2) and cultured for 2 h and then extracted with SDS-sample buffer. The cell extracts were used for Western blot with specific antibodies as indicated. *P-*, phosphorylated.

ation at Thr^{389} and Thr^{421}/Ser^{424} in "UV responses" may play a role in UV-induced carcinogenesis. Although the details of the molecular mechanisms by which H_2O_2 initiates the signal transduction pathways leading to $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} are not yet clear, it appears that H_2O_2-mediated $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} by UV radiation is through a rapamycin-sensitive, PI3K-dependent, Akt- and aPKC-independent pathway. It has been reported that PDK1 and NEK6/7 are involved in regulation of $p70^{S6k}$ phosphorylation in cell response to insulin (51, 52). It has also been reported that osmotic stress inhibits $p70^{S6k}$ through activation of a calyculin-sensitive protein phosphatase (22). Thus, we will test whether these enzymes are involved in UV-induced $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} in our future studies. Further study of the precise mechanism by which UV radiation and H_2O_2 triggers signal transduction cascades leading to $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} should help us in understanding the basis of UV-induced skin diseases, such as cancer and aging.

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REFERENCES

1. Parrish, J. A. *In:* L. A. Goldsmith, (ed.), Biochemistry and Physiology of the Skin, Vol. II, pp. 713–333. New York: Oxford University Press, 1993.
2. Epstein, J. H. Photocarcinogenesis: a review. Natl. Cancer Inst. Monogr., *50:* 13–25, 1978.
3. Stenback, F. Species-specific neoplastic progression by ultraviolet light on the skin of rats, guinea pigs, hamsters and mice. Oncology, *31:* 209–225, 1975.
4. Strickland, P. T. Photocarcinogenesis by near-ultraviolet (UVA) radiation in Sencar mice. J. Investig. Dermatol., *87:* 272–275, 1986.
5. Staberg, B., Wolf, H. C., Klemp, P., Poulsen, T., and Bradhagen, H. The carcinogenic effect of UVA irradiation. J. Investig. Dermatol., *81:* 517–519, 1983.

6. Halliwell, B., and Aruoma, O. I. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett., *281:* 9–19, 1991.
7. Radler-Pohl, A., Sachsenmaier, C., Gebel, S., Auer, H-P., Bruder, J. T., Rapp, U., Angel, P., Rahmsdorf, H. J., and Herrlich, P. UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-1 kinase. EMBO J., *12*: 1005–1012, 1993.
8. Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell, *71:* 1081–1091, 1992.
9. Devary, Y., Rosette, C., DiDonato, J. A., and Karin, M. NF-κB activation by ultraviolet light not dependent on a nuclear signal. Science (Wash. DC), *261:* 1442–1445, 1993.
10. Huang, C., Ma, W-Y., Bowden, G. T., and Dong, Z. Ultraviolet B-induced activated protein-1 activation does not require epidermal growth factor receptor but is blocked by a dominant negative PKCλ/ι. J. Biol. Chem., *49:* 31262–31268, 1996.
11. Huang, C., Ma, W-Y., and Dong, Z. Signal transduction through atypical PKCs, but not the EGF receptor, is necessary for UVC-induced AP-1 activation in immortal murine cells. Oncogene, *14:* 1945–1954, 1997.
12. Huang, C, Li, J., Chen, N., Ma, W-Y., Bowden, G. T., and Dong, Z. Inhibition of atypical PKC blocks ultraviolet-induced AP-1 activation by specifically inhibiting ERKs activation. Mol. Carcinog., *27:* 65–75, 2000.
13. Susa, M., Olivier, A. R., Fabbro, D., and Thomas, G. EGF induces biphasic S6 kinase activation: late phase is protein kinase C-dependent and contributes to mitogenicity. Cell, *57:* 817–824, 1989.
14. Reinhard, C., Thomas, G., and Kozma, S. C. A single gene encodes two isoforms of the p70 S6 kinase: activation upon mitogenic stimulation. Proc. Natl. Acad. Sci. USA, *89:* 4052–4056, 1992.
15. Proud, C. G. p70 S6 kinase: an enigma with variations. Trends Biochem. Sci., *21:* 181–185, 1996.
16. Han, J. W., Pearson, R. B., Dennis, P. B., and Thomas, G. Rapamycin, wortmannin, and the methylxanthine SQ20006 inactivate p70s6k by inducing dephosphorylation of the same subset of sites. J. Biol. Chem., *270:* 21396–21403, 1995.
17. Pearson, R. B., Dennis, P. B., Han, J. W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E., and Thomas, G. The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. EMBO J., *14:* 5279–5287, 1995.
18. Ferrari, S., Bannwarth, W., Morley, S. J., Totty, N. F., and Thomas, G. Activation of p70s6k is associated with phosphorylation of four clustered sites displaying Ser/Thr-Pro motifs. Proc. Natl. Acad. Sci. USA, *89:* 7282–7286, 1992.
19. Shepherd, P. R., Withers, D. J., and Siddle, K. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem. J., *333* (Pt. 3)*:* 471–490, 1998.
20. Toker, A. Protein kinases as mediators of phosphoinositide 3-kinase signaling. Mol. Pharmacol., *57:* 652–658, 2000.
21. Bae, G. U., Seo, D. W., Kwon, H. K., Lee, H. Y., Hong, S., Lee, Z. W., Ha, K. S., Lee, H. W., and Han, J. W. Hydrogen peroxide activates p70(S6k) signaling pathway. J. Biol. Chem., *274:* 32596–32602, 1999.
22. Parrott, L. A., and Templeton, D. J. Osmotic stress inhibits p70/85 S6 kinase through activation of a protein phosphatase. J. Biol. Chem., *274:* 24731–24736, 1999.
23. Jiang, B. H., Aoki, M., Zheng, J. Z., Li, J., and Vogt, P. K. Myogenic signaling of phosphatidylinositol 3-kinase requires the serine-threonine kinase Akt/protein kinase B. Proc. Natl. Acad. Sci. USA, *96:* 2077–2081, 1999.
24. Huang, C., Ma, W-Y., Li, J., Hecht, S. S., and Dong, Z. Essential role of p53 in phenethyl isothiocyanate (PEITC)-induced apoptosis. Cancer Res., *58:* 4102–4106, 1998.
25. Huang, C., Ma, W-Y., and Dong, Z. Resveratrol suppresses the cell transformation and induces apoptosis through p53-dependent pathway. Carcinogenesis (Lond.), *20:* 237–242, 1999.
26. Huang, C., Ma, W-Y., Hanenberger, D., Clery, M. P., Bowden, G. T., and Dong, Z. Inhibition of ultraviolet B (UVB)-induced AP-1 activity by aspirin in AP-1-luciferase transgenic mice. J. Biol. Chem., *272:* 26325–26331, 1997.
27. Ye, J., Wang, S. Leonard, S. S., Sun, Y., Butterworth, L., Antonini, J., Ding, M., Rojanasakul, Y., Vallyathan, V., Castranova, V., Shi, X. Role of reactive oxygen species and p53 in chromium (VI)-induced apoptosis. J. Biol. Chem., *274:* 34974–34980, 1999.
28. Huang, C., Zhang, Z., Ding, M., Li, J., Ye, J., Leonard, S. S., Shen, H. M., Butterworth, L., Lu, Y., Costa, M., Rojanasakul, Y., Castranova, V., Vallyathan, V., and Shi, X. Vanadium induces p53 transactivation through reactive oxygen species and causes apoptosis. J. Biol. Chem., *275:* 32516–32522, 2000.
29. Shi, X., and Dalal, N. S. Hydroxyl radical generation in the NADH/microsomal reduction of vanadate. Free Radical Res. Commun., *17:* 369–376, 1992.
30. Huang, C., Ma, WY., Li, J., and Dong, Z. Arsenic induces apoptosis through a c-Jun NH_2-terminal kinase-dependent, p53-independent pathway. Cancer Res., *59:* 3053–3058, 1999.
31. Brenneisen, P., Wenk, J., Wlaschek, M., Krieg, T., and Scharffetter-Kochanek, K. Activation of p70 ribosomal protein S6 kinase is an essential step in the DNA damage-dependent signaling pathway responsible for the ultraviolet B-mediated increase in interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) protein levels in human dermal fibroblasts. J. Biol. Chem., *275:* 4336–4344, 2000.
32. Huang, C., Li, J., Ding, M., Leonard, S. S., Wang, L., Castranova, V., Vallyathan, V., and Shi, X. UVC Induces phosphorylation of protein kinase B (Akt) at Ser-473 and Thr-308 in mouse epidermal Cl 41 cells through hydrogen peroxide. J. Biol. Chem., *276:* 40234–40240, 2001.
33. Nath, K. A., Ngo, E. O., Hebbel, R. P., Croatt, A. J., Zhou, B., and Nutter, L. M. α-Ketoacids scavenge H2O2 *in vitro* and *in vivo* and reduce menadione-induced DNA injury and cytotoxicity. Am. J. Physiol., *268:* C227–C236, 1995.
34. Tuttle, S. W., Varnes, M. E., Mitchell, J. B., and Biaglow, J. E. Sensitivity to chemical oxidants and radiation in CHO cells line deficient in oxidative pentose cycle activity. Int. J. Radiat. Oncol. Biol. Phys., *22:* 671–675, 1992.
35. Halliwell, B., and Gutteridge, J. M. C. Free Radicals in Biology and Medicine, Ed. 3, pp. 134–245. Oxford: Oxford University Press, 2000.
36. Diaz-Meco, M. T., Berra, E., Municio, M. M., Sanz, L., Lozano, J., Dominguez, I., Diaz-Golpe, V., Lain de Lera, M. T., Alcami, J., Paya, C. V., Arenzana-Seisdedos, F., Virelizier, J., and Moscat, J. A dominant negative protein kinase Cζ subspecies blocks NF-κB activation. Mol. Cell. Biol., *13:* 4770–4775, 1993.
37. Shackelford, R. E., Kaufmann, W. K., and Paules, R. S. Oxidative stress and cell cycle checkpoint function. Free Radical Biol. Med., *28:* 1387–1404, 2000.
38. Kiningham, K. K., Oberley, T. D., Lin, S., Mattingly, C. A., and St. Clair, D. K. Overexpression of manganese superoxide dismutase protects against mitochondrial-initiated poly(ADP-ribose) polymerase-mediated cell death. FASEB J., *13:* 1601–1610, 1999.
39. Buschmann, T., Yin, Z., Bhoumik, A., and Ronai, Z. Amino-terminal-derived JNK fragment alters expression and activity of c-Jun, ATF2, and p53 and increases H_2O_2-induced cell death. J. Biol. Chem., *275:* 16590–16596, 2000.
40. Adler, V., Yin, Z., Tew, K. D., and Ronai, Z. Role of redox potential and reactive oxygen species in stress signaling. Oncogene, *18:* 6104–6111, 1999.
41. Lander, H. M. An essential role for free radicals and derived species in signal transduction. FASEB J., *11:* 118–124, 1997.
42. Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. A model for p53-induced apoptosis. Nature (Lond.), *389:* 300–305, 1997.
43. Huang, C., Ma, W-Y., and Dong, Z. Requirement for phosphatidylinositol 3-kinase in epidermal growth factor-induced AP-1 transactivation and transformation in JB6 P+ cells. Mol. Cell. Biol., *16:* 6427–6435, 1996.
44. Sen, C. K., and Packer, L. Antioxidant and redox regulation of gene transcription. FASEB J., *10:* 709–720, 1996.
45. Klippel, A., Reinhard, C., Kavanaugh, W. M., Apell, G., Escobedo, M. A., and Williams, L. T. Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. Mol. Cell. Biol., *16:* 4117–4127, 1996.
46. Weng, Q. P., Andrabi, K., Klippel, A., Kozlowski, M. T., Williams, L. T., and Avruch, J. Phosphatidylinositol 3-kinase signals activation of p70 S6 kinase *in situ* through site-specific p70 phosphorylation. Proc. Natl. Acad. Sci. USA, *92:* 5744–5748, 1995.
47. Moule, S. K., Edgell, N. J., Welsh, G. I., Diggle, T. A., Foulstone, E. J., Heesom, K. J., Proud, C. G., and Denton, R. M. Multiple signalling pathways involved in the stimulation of fatty acid and glycogen synthesis by insulin in rat epididymal fat cells. Biochem. J., *311*(Pt. 2)*:* 595–601, 1995.
48. Burgering, B. M., and Coffer, P. J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature (Lond.), *376:* 599–602, 1995.
49. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. Phosphorylation and activation of p70s6k by PDK1. Science (Wash. DC), *279:* 707–710, 1998.
50. Romanelli, A., Martin, K. A., Toker, A., and Blenis, J. p70 S6 kinase is regulated by protein kinase Cζ and participates in a phosphoinositide 3-kinase-regulated signalling complex. Mol. Cell. Biol., *19:* 2921–2928, 1999.
51. Balendran, A., Currie, R., Armstrong, C. G., Avruch, J., and Alessi, D. R. Evidence that 3-phosphoinositide-dependent protein kinase-1 mediates phosphorylation of p70 S6 kinase *in vivo* at Thr-412 as well as Thr-252. J. Biol. Chem., *274:* 37400–37406, 1999.
52. Belham, C., Comb, M. J., and Avruch, J. Identification of the NIMA family kinases NEK6/7 as regulators of the p70 ribosomal S6 kinase. Curr Biol., *11:* 1155–1167, 2001.

Ultraviolet-induced Phosphorylation of $p70^{S6K}$ at Thr^{389} and Thr^{421}/Ser^{424} Involves Hydrogen Peroxide and Mammalian Target of Rapamycin but not Akt and Atypical Protein Kinase C

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