Signal Transduction Pathways Involved in Phosphorylation and Activation of p70S6K Following Exposure to UVA Irradiation*

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SUMMARY

Ultraviolet light A (UVA) plays an important role in the etiology of human skin cancer and UVA-induced signal transduction has a critical role in UVA-induced skin carcinogenesis. The upstream signaling pathways leading to p70\(^{S6K}\) phosphorylation and activation are not well understood. Here, we observed that UVA induces phosphorylation and activation of p70\(^{S6K}\). Further, UVA-stimulated p70\(^{S6K}\) activity and phosphorylation at Thr389 were blocked by wortmannin, rapamycin, PD98059, SB202190, and dominant negative mutants of PI3-kinase p85 subunit (DNM-\(\Delta p85\)), ERK2 (DNM-ERK2), p38 kinase (DNM-p38), and JNK1 (DNM-JNK1) and were absent in \(Jnk1^{-/-}\) or \(Jnk2^{-/-}\) knockout cells. The p70\(^{S6K}\) phosphorylation at Ser411 and Thr421/Ser424 was inhibited by rapamycin, PD98059, or DNM-ERK2, but not by wortmannin, SB202190, DNM-\(\Delta p85\), or DNM-p38. However, Ser411, but not Thr421/Ser424 phosphorylation, was suppressed in DNM-JNK1 and abrogated in \(Jnk1^{-/-}\) or \(Jnk2^{-/-}\) cells. In vitro assays indicated that Ser411 on immunoprecipitated p70\(^{S6K}\) proteins is phosphorylated by active JNKs and ERKs, but not p38 kinase, and Thr421/Ser424 is phosphorylated by ERK1, but not ERK2, JNKs, or p38 kinase. Moreover, p70\(^{S6K}\) co-immunoprecipitated with PI-3 kinase and possibly PDK1. The complex possibly possessed a partial basal level of phosphorylation, but not at MAPKs sites, which was available for its activation by MAPKs in vitro. Thus these results suggest that activation of MAPKs, like PI-3 kinase/mTOR, may be involved in UVA-induced phosphorylation and activation of p70\(^{S6K}\).
INTRODUCTION

Ultraviolet light A (UVA) \(^1\) (320-400 nm) comprises approximately 95% of the total solar UV (1) because all the ultraviolet C (UVC) (200-290 nm) and most of the ultraviolet B (UVB) (290-320 nm) radiation are absorbed by the earth’s stratospheric ozone layer (2). Currently UVA, like UVB, is considered to be a complete carcinogen (3) and to play an important role in the etiology of human skin cancer (4). But, the activation of signaling molecules and their pathways implicated in the process following UVA irradiation (5, 6) are not well understood. Therefore, the study of UVA-induced signal transduction will help in understanding the molecular mechanisms underlying UVA-induced carcinogenesis.

Activation of tumor cell proliferation requires an accelerated rate of protein synthesis, which is regulated in part by intracellular activation of several signaling protein kinase cascades that interact with the translational machinery of the ribosome (7). Among them, S6 is a component of ribosomal proteins and is located at the interface between 40S and 60S ribosomal proteins (8). Phosphorylation of S6 at multiple serine sites on its C-terminus was shown to be correlated with increased translation, especially of mRNAs containing a polypyrmididine tract in their 5’ untranslated regions (5’TOP) (9). This family of mRNAs constitutes as few as 100 to 200 genes, but makes up 20% to 30% of the total cellular mRNA, indicating that they are important for cell cycle progression. The family of serine/threonine kinases that mediate S6 phosphorylation are known as ribosomal S6 kinases, one of which is a 70-kDa S6 kinase (p70\(^{S6K}\)) (8). Accumulating evidence suggests that the prominent role of p70\(^{S6K}\) activation in mitogenesis may be to promote translation of mRNAs necessary for cell growth and division and to generate many of the molecules necessary for driving the cell cycle from G0/G1 to S phase (9).
Initially p70\textsuperscript{S6K} was isolated from mitogen-stimulated Swiss mouse 3T3 cells (10). Subsequently, two isoforms of p70\textsuperscript{S6K} (p70\textsuperscript{S6K}/p85\textsuperscript{S6K}, collectively termed p70\textsuperscript{S6K} or S6K1) were found in purification, cloning, and expression studies (11). Both isoforms are encoded by the same transcript with alternative translational start sites (12). Based on evidence that an additional 23-amino acid extension at the N-terminus of p85\textsuperscript{S6K} was shown to function as a nuclear localization signal (NLS), p85\textsuperscript{S6K} appears to be exclusively nuclear, whereas p70\textsuperscript{S6K} is largely cytoplasmic (11, 12). The p85\textsuperscript{S6K} may be responsible for phosphorylation of the free chromatin-bound nuclear form of S6 (13, 14). Recently, deletion of the p70\textsuperscript{S6K} gene was shown to have no effect on S6 phosphorylation, 5'TOP mRNA translation, or the rate of cell growth, but resulted in a small mouse phenotype (15). In p70\textsuperscript{S6K-/-} mice, another S6 kinase (S6K2) with a 70% overall amino acid homology with p70\textsuperscript{S6K} and a potential NLS at the C-terminus was found to partially compensate for loss of p70\textsuperscript{S6K} function (15). Recently, another nuclear S6 kinase-related kinase (SRK) was cloned and identified as a novel nuclear target of Akt (16). Generally, the p70\textsuperscript{S6K} family plays a key role in the control of cell size, growth, and proliferation. Consistent with this concept, inhibition of p70\textsuperscript{S6K} activation by microinjection of neutralizing antibodies (17) or treatment of cells with rapamycin, an inhibitor of mammalian target of rapamycin (mTOR)-p70\textsuperscript{S6K} (18, 19, 20), severely impeded cell cycle progression.

Although p70\textsuperscript{S6K} is known to be activated by various stimuli including growth factors, cytokines, 12-\textit{O}-tetradecanoylphorbol-13-acetate (TPA), oncogenic products, Ca\textsuperscript{2+}, and inhibitors of protein synthesis (5, 21), the signal transduction pathway mediating p70\textsuperscript{S6K} is poorly understood. An array of independently regulated protein kinases (12, 22, 23) are known to activate 70\textsuperscript{S6K} via phosphorylation of at least eight Ser/Thr sites in its three separate domains. Thr229 in the p70\textsuperscript{S6K} activation loop within the catalytic domain has been shown to be
phosphorylated in vivo through the phosphatidylinositol-3 kinase (PI-3 kinase) pathway (24) and in vitro selectively by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (25). Thr229 phosphorylation has been shown to enable p70S6K activity and be repressed by wortmannin, an inhibitor of PI-3 kinase (26). Additionally, similar to Thr229, Ser371 located in the kinase extension domain, has been shown to influence p70S6K activity and its phosphorylation is also regulated by the PI-3 kinase-dependent pathway (27). Thr389 is another site for mitogen-stimulated phosphorylation and is situated in a conserved 65-amino acid segment located immediately C-terminal to the catalytic domain (12, 22). It plays an especially important role in p70S6K activation because it influences both the phosphorylation of Thr229 in vitro by PDK1 and p70S6K activity (22). Although p70S6K was shown to be phosphorylated by mTOR in vitro (28, 71), a p70S6K∆2-46/∆CT104 construct was shown to still be activated and phosphorylated at Thr389 in vivo in the presence of rapamycin, an mTOR inhibitor (12, 22, 29). Other studies indicated that mTOR activity likely suppresses protein phosphatase 2A-mediated dephosphorylation of p70S6K (30, 31, 32).

At the same time that p70S6K is activated by mitogens, the extracellular signal-regulated kinases (ERKs) pathway is also stimulated (23), indicating that the activation of p70S6K appears to coincide with that of ERKs. The proline-directed Ser/Thr (S/T-P) sites (Ser411, Ser418, Thr421, and Ser424) within the autoinhibitory domain of p70S6K are in a consensus motif similar to those known to serve as recognition determinants for mitogen-activated protein kinases (MAPKs). Indeed, ERKs were shown to phosphorylate p70S6K in vitro, suggesting that the Ras/ERK pathway controlled p70S6K activation (33); however, other studies (34, 35) showed that ERKs were neither necessary nor sufficient for p70S6K activation. But more recently, the ERKs cascade, like PI-3 kinase/Akt cascades, has been demonstrated to be a prerequisite for p70S6K
activation (23, 36). Taken together, p70S6K activation appears to require a complex array of separate, concurrent phosphorylations at multiple sites catalyzed by various protein kinases, but its precise mechanisms of activation are as yet unclear. In our work, we provide evidence that MAPK pathways, like the PI-3 kinase/mTOR pathways, are implicated in phosphorylation and activation of p70S6K in response to UVA irradiation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Chemicals were the best grades available commercially. Eagle’s minimum essential medium (MEM) and fetal bovine serum (FBS) were from Whittaker Biosciences, Inc. (Walkersville, MD); Dulbecco’s modified Eagle’s medium (DMEM), L-glutamine, gentamicin, and G418 sulfate were from GibcoBRL Life Technologies, Inc. (Grand Island, NY); aprotinin, leupeptin, TPA (12-O-tetradecanoylphorbol-13-acetate), PD98059, SB202190, and rapamycin were purchased from Sigma (St. Louis, MO); wortmannin was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA); PD169316 was from Alexis® Biochemicals, Inc. (San Diego, CA); and epidermal growth factor (EGF) was from Collaborative Research (Madison, WI). The phospho-specific antibodies against phosphorylated sites of ERKs (Tyr204 of p44 and p42), c-Jun N-terminal kinases (JNKs) (Thr183/Tyr185), p38 kinase (Thr180/Tyr182), and antibodies to nonphospho-ERKs, -JNKs, -p38 kinase were from New England BioLabs Inc. (Beverly, MA). The polyclonal antibodies against phospho-p70S6K at Ser411, Thr421/Ser424, or Thr389 and anti-nonphospho-70S6K antibodies were also from New England BioLabs Inc. Mouse anti-phospho-specific p70S6K (Ser411) mouse monoclonal antibody (A-6) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Active ERK1, ERK2, JNK1, JNK2,
and p38 kinase and p70\textsuperscript{S6K} S6 activity assay kits were purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

**Stable Transfectants and Cell Culture**—The CMV-neo vector plasmid was constructed as previously reported (37). Mouse epidermal JB6 promotion sensitive Cl 41 and its stable transfectants with CMV-neo mass\textsubscript{1} (Cl 41) (38) or with dominant negative mutants of ERK2 (DNM-ERK2) (39), JNK1 (DNM-JNK1) (38), p38 kinase (DNM-p38) (40), or PI-3 kinase p85 subunit (DNM-Δp85) (41) were established as previously reported and cultured in monolayers using Eagle’s MEM supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine, and 25 µg/ml gentamicin at 37 °C in humidified air with 5% CO\textsubscript{2}. Before each experiment, the transfectants were selected with G418 and tested with their phospho-specific MAPKs antibodies.

**Treatment of Cells with UVA, UVB, UVC or Kinase Inhibitors**—The UVA source used was a Philips TL100w/10R system from Ultraviolet Resources International (Lakewood, OH). It consists of a Magnetek transformer number 799-XLH-TC-P, 120 volts 60 hertz, and six bulbs each six feet long. UVA irradiation filtered through about 6 mm of plate glass, eliminating most of UVB and UVC light at all wavelengths below 320 nm, was performed on cultured cells in the UVA box with two ventilation fans installed to eliminate thermal stimulation. These adjustments were necessary because the normal UVA lamps can also produce a small amount of UVB and UVC. The UVB irradiation was carried out in a UVB chamber with a transluminator emitting UVB light protons and was fitted with a Kodak Kodacel K6808 filter that eliminates all wavelengths below 290 nm. This was also necessary because a normal UVB lamp can generate a small number of UVC light protons. UVC radiation performed was from germicidal lamps. To assess the roles of different signaling pathways in p70\textsuperscript{S6K} phosphorylation, we pretreated JB6 Cl
41 cells for 1 to 2 h before UVA irradiation with dimethyl sulfoxide (DMSO) or kinase inhibitors including PD98059, SB202190, PD169316, wortmannin, or rapamycin dissolved in DMSO.

**Phosphorylation of ERKs, JNKs and p38 Kinase**—Immunoblot analysis for detection of phosphorylated proteins for ERKs, JNKs, and p38 kinase was carried out using the phospho-specific MAPK antibodies as reported previously (38, 39, 40, 41). The antibody-bound protein complexes were detected by western immunoblotting using a chemiluminescent detection system (ECL, New England BioLabs Inc.). Some transfer membranes were washed with stripping buffer (7 M guanidine hydrochloride, 50 mM glycine pH 10.8, 0.05 mM EDTA, 0.1 M KCl and 20 mM β-mercaptoethanol) and reprobed with other primary phospho-specific or nonphospho-specific antibodies.

**Analysis of In Vivo Phosphorylation of p70^S6K with Phospho-specific Antibodies**—Cells (40 x 10^4 to 80 x 10^4) were seeded into 100-mm dishes and cultured for 24 to 48 h until the cells reached 80% to 90% confluence. The CI 41, DNM-ERK2, DNM-JNK1, DNM-p38, or DNM-Δp85 cells were starved for 24 to 48 h in MEM containing 0.1% FBS, 2 mM L-glutamine, and 25 µg/ml gentamicin. After treatment with UVA or kinase inhibitors as indicated (prior to irradiation), the cells were washed once with ice-cold phosphate buffered saline (PBS) and lysed in 200 µl of SDS sample lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM dithiothreitol (DTT) and 0.1% bromophenol blue. The lysed samples were scraped into 1.5-ml tubes and sonicated for 5 to 10 s. Samples containing equal amounts of protein (Bio-Rad protein assay, Bio-Rad Laboratories Inc., Hercules, CA), were loaded into each lane of an 8% SDS polyacrylamide gel for electrophoresis (SDS-PAGE) and subsequently transferred onto an Immobilon-p transfer membrane. The phosphorylated p70^S6K protein was selectively detected by western immunoblotting using a chemiluminescent detection system and
phospho-specific antibodies against p70S6K phosphorylation at Ser411, Thr421/Ser424, or Thr389. Nonphosphorylated p70S6K was used as a control to verify equal protein loading.

**p70S6K Activity Assay**—p70S6K activity was measured by an immune complex kinase assay using an S6 peptide AKRRRLSSLRA as a substrate according to the procedure recommended in the S6 kinase assay kit (Upstate Biotechnology Inc.) (5). Briefly, cell lysates were prepared from JB6 Cl 41 cells or Cl 41 cell lines expressing DNM-ERK2, DNM-JNK1, DNM-p38, or DNM-Δp85 grown in 100-mm dishes. After starving by replacing media with 0.1% FBS-MEM, the cells were or were not pretreated with inhibitors as described above and then irradiated with UVA (160 kJ/m²). The cells were harvested at the times indicated and lysed in 300 μl of buffer A containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF. The cell lysates were clarified by centrifugation at 17,000 g for 5 min at 4 °C. The supernatant fractions containing equal amounts of proteins were incubated with p70S6K antibody at 4 °C overnight and then for an additional 4 h with protein A/G sepharose beads (Santa Cruz Biotechnology Inc.). After washing four times with PBS, the immunoprecipitates were incubated at 30 °C for 10 min in a mixture of the following: 20 μl of assay dilution buffer (ADB: 20 mM MOPS, pH 7.2, 25 mM β–glycerol phosphate, 5 mM EGTA, 1 mM Na₃VO₄ and 1 mM DTT), 10 μl of substrate cocktail (S6 peptide in ADB), 10 μl of inhibitor cocktail (20 μM PKC inhibitor peptide, 2 μM protein kinase A inhibitor peptide and 20 μM compound R24571 in ADB), and 10 μl of [γ³²P] ATP (1 μCi/μl; Amersham Pharmacia Biotech Inc., Piscataway, NJ). To stop the reaction, each sample was spotted onto a numbered P81 paper square and washed three times (5 min each) with 0.75% phosphoric acid and once (5 min) with acetone. Each sample paper was transferred into a
scintillation vial containing 5 ml of scintillation fluid and then counted in a β-scintillation counter. At the same time, immunoprecipitates incubated non-immune IgG serum instead of the p70\text{S6K}\textsuperscript{\textregistered} antibody were used as background controls. After subtraction of background from each of the samples, the UVA-stimulated p70\text{S6K}\textsuperscript{\textregistered} kinase activity was normalized to unstimulated controls and expressed as fold change.

**Preparation of Normal, Jnk1-/-, and Jnk2-/- Primary Embryo Fibroblasts**—Embryonic fibroblasts from normal, Jnk1-/- and Jnk2-/- knockout mice were isolated and prepared according to the procedure of Loo and Cotman (42). Cells were established in culture in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO\textsubscript{2} at 37 °C. For analysis of protein phosphorylation, the cells were starved by replacing growth medium with serum-free DMEM for 12 h at which time cells were exposed to UVA. The cells were lysed with SDS sample buffer and the protein concentration in the supernatant fraction of the cell lysates was determined (Bio-Rad assay). Equal amounts of protein were resolved by 8% SDS-PAGE and phosphorylated and nonphosphorylated p70\text{S6K}\textsuperscript{\textregistered} proteins were determined by western blotting analysis. Additionally, p70\text{S6K}\textsuperscript{\textregistered} kinase activity in these cell lines was measured following immunoprecipitation procedures as described above.

**In Vitro Kinase Assay for p70\text{S6K}\textsuperscript{\textregistered} Phosphorylation and Activation**—JB6 Cl 41 cells were cultured in 100-mm dishes, starved for 24 h, and then lysed in buffer A alone. The cell lysates were centrifuged and then the supernatant fractions were subjected to immunoprecipitation with rabbit anti-nonphosphorylated p70\text{S6K}\textsuperscript{\textregistered} polyclonal antibody as described above. Samples containing immunoprecipitated p70\text{S6K}\textsuperscript{\textregistered} were incubated with active ERK1, ERK2, JNK1, JNK2, or p38 kinases (Upstate Biotechnology Inc.) at doses as indicated in kinase buffer (50 mM Tris-
HCl pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 5 mM ATP and 0.01 % Brij 35) (New England BioLabs Inc.) at 30 °C for 60 min. The reactions were stopped by adding SDS sample buffer and phosphorylation of immunoprecipitated p70S6K protein was analyzed by using SDS-PAGE, western blotting and a chemiluminescent detection system. The first antibodies are mouse phospho-specific p70S6K (Ser411) monoclonal antibody (Santa Cruz Biotechnology Inc.) and rabbit phospho-specific p70S6K (Thr389, Thr421/Ser424) polyclonal antibodies (New England BioLabs Inc.). To further analyze whether p70S6K is activated by MAPKs in vitro, samples containing immunoprecipitated p70S6K were incubated at 30 °C for 30 min with S6 peptide plus active ERK1 (10 ng/µl), ERK2 (10 ng/µl), JNK1 (25 mU/µl), JNK2 (25 mU/µl), or p38 kinases (10 ng/µl) (Upstate Biotechnology Inc.) and p70S6K kinase activity was determined as described above. At the same time, incubation of immunoprecipitated p70S6K with S6 peptide only was used as a negative control and incubations of S6 peptide with MAPKs were used as internal controls. Addition of BSA instead of MAPKs or immunoprecipitated p70S6K proteins were used as background controls.

Determination of p70S6K Co-immunoprecipitates—After starvation for 48 h, JB6 Cl 41 cells were or were not irradiated with UVA at 160 kJ/m² and then harvested at 15 or 30 min following irradiation. Immunoprecipitated p70S6K proteins were obtained by incubating the cell lysates with p70S6K antibody as described above. At the same time, immunoprecipitates with normal non-immune IgG serum instead of p70S6K antibody were used as internal negative controls and immunoprecipitates with antibodies against PI-3 kinase p85α (Z-8), p90 ribosomal S6 kinases (RSK)3 (C-20), and phospho-tyrosine (PY99 from Santa Cruz Biotechnology Inc.), PDK1, Akt, RSK1, RSK2, and mitogen- and stress-activated protein kinases (MSK)1 (Upstate Biotechnology Inc.) were used as positive controls. To examine whether p70S6K co-
immunoprecipitates with PI-3 kinase, immunoprecipitated p70S6K proteins and corresponding PI-3 kinase controls were incubated with phosphatidylinositol 4,5-diphosphate (Sigma), a preferential substrate of PI-3 kinase, and then PI-3 kinase activity was determined by running thin-layer chromatography plates as described below. To further test whether p70S6K co-immunoprecipitates with the other above-mentioned kinases, immunoprecipitated p70S6K proteins and positive controls were subjected to SDS-PAGE and western immunoblotting with corresponding nonphosphorylated or phospho-specific antibodies according to the procedure as described above.

**PI-3 Kinase Assay**—PI-3 kinase activity was determined according to reported methods (43,44). Briefly, JB6 Cl 41 cells or JB6 cells expressing DMN-Δp85 were cultured in monolayers in 100-mm dishes. Then cells were starved for 24 h in serum-free MEM. At 1.5 h following pretreatment with or without wortmannin at doses indicated, the cells were irradiated with UVA at 160 kJ/m². After an additional incubation for 15 min at 37 °C, the cells were washed once with ice-cold PBS and lysed in buffer B (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM MgCl₂, 10% glycerol, 1% Nonidet P-40, 1 mM DTT, 1 mM Na₃VO₄, 1 mM PMSF, 10 µg/ml of aprotinin, leupetin and pepstatin). The lysates were centrifuged and the supernatant fractions containing equal amounts of protein were incubated overnight at 4 °C with 20 µl of agarose beads previously conjugated with mouse monoclonal phosphotyrosine antibody (PY99) or with rabbit polyclonal PI-3 kinase p85α antibody (Z-8). The immunocomplex-bound beads were washed twice with each of the following buffers: 1) PBS containing 1% Nonidet P-40, 1 mM DTT and 0.1 mM Na₃VO₄; 2) 100 mM Tris-HCl, pH 7.6, 0.5 M LiCl, 1 mM DTT and 0.1 mM Na₃VO₄; 3) 10 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM DTT and 0.1 mM Na₃VO₄. The beads were incubated for 5 min on ice in 20 µl of buffer 3, and then 20 µl of 0.5 mg/ml
phosphatidylinositol 4,5-diphosphate previously sonicated in substrate buffer (50 mM HEPES, pH 7.6, 1 mM EGTA, 1 mM NaH$_2$PO$_4$) was added. After incubation for 5 min at room temperature, 10 µl of the reaction buffer (10 mM Tris-HCl, pH 7.6, 60 mM MgCl$_2$, 250 µM ATP containing 10 µCi of [$\gamma$-32p]ATP) was added and then the beads were incubated for an additional 10 min at 30 °C. The reaction was stopped by addition of 15 µl of 4 N HCl and subsequent 130 µl of chloroform/methanol (1:1, v/v). After vortexing for 30 s, 30 µl of lower phospholipid-containing chloroform phase was spotted onto thin-layer chromatography plates coated with Silica Gel H containing 1% potassium oxalate (Analtech Inc., Newark, DE), that were baked at 110 °C for at least 1 h before use. The plates were developed in tanks containing chloroform/methanol/ NH$_4$OH/H$_2$O (60:47:2:11.3, v/v) until the solvent reached the top of the plates. The plates were dried at room temperature and autoradiographed. The PIP3 phosphate blotting was quantified by the Phosphor-Image software (Molecular Dynamics, Sunnyvale, CA).

**Kinase Activity Assay for Protein Phosphatase 1-treated p70$^{S6K}$ Preparations**—Deactivation of the p70$^{S6K}$ preparations by protein phosphatase 1 (PP1) and reactivation by MAPKs were examined according to reported methods (72, 73). Briefly, JB6 Cl 41 cells were starved for 36 h and then lysed in buffer A alone. The cell lysates were clarified by centrifugation at 17,000 g at 4 °C for 5 min and then supernatant fractions containing equal amounts of protein were subjected to immunoprecipitation with p70$^{S6K}$ antibody as described above. The p70$^{S6K}$ preparations were incubated at 30 °C for 30 min with 0.5 unit of PP1 or with no PP1 (as internal controls) in 100 µl buffer containing 50 mM Tris-HCl (pH 7.0), 30 mM β-mercaptoethanol, 0.01% Brij-35 (Sigma). Then, PP1 activity was inhibited by the addition of NaF (10 mM). Deactivated p70$^{S6K}$ preparations were saved by centrifugation and then incubated at 30 °C for 30 min with ERK1 (10 ng/µl), ERK2 (10 ng/µl) (New England BioLabs Inc.), JNK1
(50 mU/µl), JNK2 (50 mU/µl), p38 kinase (10 ng/µl) (Upstate Biotechnology Inc.), and subsequent p70\text{S6K} S6 kinase activity assay was performed as described above.

**Statistical Analysis**—Significant differences between the p70\text{S6K} kinase activity after treatment and the corresponding control value were determined using the Student’s t test.

**RESULTS**

**UVA Induces Activation of p70\text{S6K} in a Time-dependent Manner**—Activation of p70\text{S6K} was reported to occur through multi-site phosphorylations (12, 22, 23). We investigated the effect of UVA on total p70\text{S6K} kinase activity and found that UVA (160 kJ/m\textsuperscript{2}) stimulated p70\text{S6K} kinase activity in a time-dependent manner (Fig. 1A; p<0.001). The activity peaked 30 min after irradiation and then decreased by 60 min. UVA-activated p70\text{S6K} kinase activity was about 3 times that of the unstimulated control 30 min following irradiation. Recently, UVB was shown to induce p70\text{S6K} activation (30, 45) and here, UVB (8 kJ/m\textsuperscript{2}) was used as a positive control and stimulated p70\text{S6K} activity about 2.3 times compared to the control (Fig. 1A).

**UVA Induces Phosphorylation of p70\text{S6K}**—TPA and EGF are well-known tumor promoters that act during tumor promotion and progression (46, 47) and UVC is also an experimental carcinogen (48). TPA, EGF, and UVC were reported to induce p70\text{S6K} phosphorylation (5, 30, 49, 50) and were therefore used as positive controls in this study. Our data showed that UVA (80 and 160 kJ/m\textsuperscript{2}) induced phosphorylation of p70\text{S6K} at Ser411, Thr421/Ser424 and Thr389. The phosphorylation was at least equivalent to or greater than that induced by UVC (60 kJ/m\textsuperscript{2}), TPA (10 ng/ml), or EGF (100 ng/ml) (Fig. 1B).

**UVA-induced Phosphorylation of p70\text{S6K} is Dose- and Time-dependent**—UVA induced phosphorylation of p70\text{S6K} Ser411, Thr389, and Thr421/Ser424 in a dose- (Fig. 2A) and time-
(Fig. 2B) dependent manner. Maximum stimulation occurred at all four sites with a UVA dose of 160 kJ/m² after 30 min followed by a decreased level by 120 min. However, phosphorylation at Ser411 and Thr421/Ser424, but not Thr389, showed a biphasic pattern with the first peak occurring at 30 min and a second peak occurring at 360 min following UVB irradiation (Fig. 2B). The time course pattern of phosphorylation coincides very well with the pattern of p70^{S6K} kinase activity (Fig. 1A), suggesting that phosphorylation of p70^{S6K} may indirectly reflect p70^{S6K} kinase activity.

**Wortmannin and DNM-Δp85 Inhibit UVA-induced Activation and Phosphorylation of p70^{S6K} at Thr389 but not at Ser411 or Thr421/Ser424**—Studies have shown that PI-3 kinase and its downstream kinases, protein kinase B (PKB)/Akt and PDK1, are required for activation and phosphorylation of p70^{S6K} (12). Here, we assess the role of the PI-3 kinase pathway in UVA-stimulated activation and phosphorylation of p70^{S6K} at Thr389, Ser411, and Thr421/Ser424. Our data showed that UVA-induced phosphorylation of p70^{S6K} at Thr389, but not at Ser411 or Thr421/Ser424, was inhibited by pretreatment of JB6 cells with wortmannin (Fig. 3A), a selective inhibitor of the PI-3 kinase p110 subunit (51). We also observed that wortmannin markedly suppressed activation of PI-3 kinase (Fig. 3B) and p70^{S6K} (Fig. 3C; p<0.001) in response to UVA irradiation. Further, we used a JB6 cell line expressing a dominant negative mutant of PI-3 kinase p85 subunit (DNM-Δp85) (41) to study the role of the PI-3 kinase pathway in UVA-stimulated phosphorylation and activation of p70^{S6K}. As expected, PI-3 kinase activity induced by UVA was significantly lower in DNM-Δp85 cells (Fig. 3E), agreeing with the results of Sajan et al. (52) who showed that DNM-Δp85 blocks p85 subunit-dependent PI-3 kinase and related pathway activation. Following UVA irradiation, phosphorylation of p70^{S6K} at Thr389, but not at Ser411 or Thr421/Ser424 (Fig. 3D), and p70^{S6K} kinase activity was almost completely
attenuated (p<0.001) in DNM-Δp85 cells compared to control Cl 41 cells expressing only CMV-neo vector (Fig. 3F). Therefore, these results indicate that the PI-3 kinase pathway may be required for UVA-induced p70S6K activation and phosphorylation at Thr389, but not for phosphorylation at Ser411 or Thr421/Ser424. This suggests that phosphorylation of p70S6K at Ser411 and Thr421/Ser424 appears to occur through activation of PI-3 kinase-independent pathways.

**Rapamycin Blocks UVA-induced Activation and Phosphorylation of p70S6K at all Four Sites**—Many experiments have shown that the mTOR signaling pathway is involved in activation and phosphorylation of p70S6K (12, 22, 28, 31, 32). Our data showed that UVA-induced phosphorylation of p70S6K at Thr389 was completely abrogated by pretreatment with rapamycin (Fig. 4A), a selective mTOR inhibitor that abolishes mTOR function both in vivo and in vitro (12, 53). UVA-induced phosphorylation of p70S6K at Ser411 and Thr421/Ser424 also was partially blocked by rapamycin pretreatment (Fig. 4A). Furthermore, our data demonstrated that p70S6K kinase activation by exposure to UVA irradiation was significantly (p<0.01) suppressed by rapamycin pretreatment (Fig. 4B). These results suggest that mTOR may preferentially mediate p70S6K phosphorylation at Thr389 leading to its activation.

**PD98059 and DNM-ERK2 Inhibit UVA-induced Activation and Phosphorylation of p70S6K at All Four Sites**—ERKs were reported to phosphorylate p70S6K in vitro (33), but later reports indicated that p70S6K activation was independent of the Ras/MAPK pathway (34, 35). However, more recently, ERKs were shown to be required for activation of p70S6K in vivo (22, 23). In our present experiments, we used PD98059, an inhibitor of MEK1 (50, 54) to determine the role of ERKs in activation and phosphorylation of p70S6K at Thr389, Ser411, and Thr421/Ser424. Our data showed that pretreatment of JB6 Cl 41 cells with PD98059 markedly
blocked UVA-induced ERKs phosphorylation, p70S6K phosphorylation at Thr421/Ser424 and Thr389, and partially inhibited phosphorylation of p70S6K at Ser411 (Fig. 5A). Further, UVA-stimulated p70S6K kinase activity was also significantly suppressed (p<0.001) by PD98059 pretreatment (Fig. 5C). However, Kamakura et al. (55) reported that PD98059 inhibits EGF-induced ERK5 activation. Therefore, to further study the role of ERKs in the phosphorylation and activation of p70S6K, we used a JB6 cell line stably expressing the protein of a dominant negative mutant of ERK2 (DNM-ERK2) (39). As expected, UVA-induced phosphorylation and activation of ERKs were blocked by DNM-ERK2 (Fig. 5B and data not shown). Our data also showed that following UVA irradiation p70S6K phosphorylation at Thr421/Ser424 and Thr389 was almost completely blocked and the phosphorylation at Ser411 was partially inhibited in DNM-ERK2 cells compared to control Cl 41 cells (Fig. 5B). Moreover, p70S6K kinase activity induced by UVA was significantly (p<0.001) abrogated in DNM-ERK2 cells compared to control cells (Fig. 5D). On the other hand, PD98059 pretreatment (Fig. 5A) and DNM-ERK2 expression (Fig. 5B) had no effect on UVA-induced p38 kinase phosphorylation. Thus, these results suggest that ERKs may be involved in p70S6K activation and phosphorylation at Thr421/Ser424, Thr389 and Ser411 in JB6 cells exposed to UVA irradiation.

SB202190 and DNM-p38 inhibit UVA-induced p70S6K Activation and Phosphorylation at Thr389 but not Ser411 or Thr421/Ser424—To determine whether p38 kinase has an effect on activation and phosphorylation of p70S6K, we employed SB202190, a selective inhibitor of p38 kinase (56). Our data showed that pretreatment of JB6 cells with SB202190 prevented phosphorylation of p38 kinase and p70S6K at Thr389, but had no effect on ERKs and p70S6K phosphorylation at Ser411 or Thr421/Ser424 following UVA irradiation (Fig. 6A). We observed that SB202190 pretreatment also significantly (p<0.01) suppressed p70S6K activation after cells

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were exposed to UVA irradiation (Fig. 6C). To further investigate the role of p38 kinase in UVA-stimulated activation and phosphorylation of \( p70^{S6K} \), we used a JB6 cell line expressing a dominant negative mutant of p38 kinase (DNM-p38) (40). As expected, DNM-p38 selectively inhibited phosphorylation and activation of p38 kinase, but not ERKs, in response to UVA (Fig. 6B and data not shown). Furthermore, DNM-p38 only blocked Thr389 phosphorylation, but had no effect on phosphorylation of \( p70^{S6K} \) at Ser411 or Thr421/Ser424, compared to corresponding control Cl 41 cells (Fig. 6B). In addition, UVA-stimulated \( p70^{S6K} \) kinase activity was suppressed (p<0.05) in DNM-p38 cells compared to control Cl 41 cells (Fig. 6D). Therefore, these results indicated that p38 kinase may be implicated in UVA-induced activation and phosphorylation of \( p70^{S6K} \) at Thr389, but not at Ser411 or Thr421/424.

**JNKs May be Involved in UVA-induced Activation and Phosphorylation of \( p70^{S6K} \) at Thr389 and Ser411 but not at Thr421/Ser424**—We found that UVA-stimulated activation of \( p70^{S6K} \) was significantly abolished by pretreatment of JB6 cells with PD169316 (Fig. 6C), a novel inhibitor of p38 kinase and JNKs (57, 58), suggesting that JNKs, like ERKs and p38 kinase, may be implicated in UVA-induced activation and phosphorylation of \( p70^{S6K} \). Further, we used cell lines with a dominant negative JNK1 mutant (DNM-JNK1) (38) and embryo \( Jnk1^{-/-} \) and \( Jnk2^{-/-} \) knockout cells to assess whether JNKs play a role in activation and phosphorylation of \( p70^{S6K} \) induced by UVA. Our data showed that UVA-induced phosphorylation of \( p70^{S6K} \) at Ser411 and Thr389, but not at Thr421/Ser424, was inhibited by DNM-JNK1 compared to control Cl 41 cells (Fig. 7A), and the phosphorylation at the same two sites was attenuated in \( Jnk1^{-/-} \) and \( Jnk2^{-/-} \) knockout cells compared to wild-type \( Jnk^{+/+} \) cells (Fig. 7B). Also, \( p70^{S6K} \) activation by UVA was partially inhibited (p<0.05) in DNM-JNK1 cells (Fig. 7C) and almost totally blocked (p<0.001) in \( Jnk1^{-/-} \) and \( Jnk2^{-/-} \) cells (Fig. 7D), as compared to those in corresponding control
cells. In addition, UVA-stimulated phosphorylation and activation of JNKs was blocked in DNM-JNK1 cells (Fig. 7A and data not shown), and corresponding phosphorylation and activation of JNKs was attenuated in Jnk1−/− or Jnk2−/− cells (Fig 7B and data not shown). These results suggest that p70S6K activation and phosphorylation at Ser411 and Thr389, but not at Thr421/Ser424, may be required for JNKs pathway activation when cells are exposed to UVA.

**Immunoprecipitated p70S6K Proteins are Phosphorylated by Active MAPKs In Vitro**—ERKs were previously shown to phosphorylate p70S6K \textit{in vitro} and to mediate p70S6K activation (33). More recently, activation of the ERKs cascade, like the PI-3 kinase/mTOR cascade, was reported to be a prerequisite for p70S6K activation (23). In our \textit{in vitro} study, immunoprecipitated p70S6K proteins from unstimulated cell lysates were phosphorylated at Ser411 when incubated with active ERK1, ERK2, JNK1, or JNK2, but not with p38 kinase (Fig. 8A). Phosphorylation at Thr421/Ser424 was induced only by ERK1 and not by the other above-mentioned MAPKs (Fig. 8A). However, the immunoprecipitated p70S6K proteins were not phosphorylated at Thr389 by \textit{in vitro} incubation with active ERKs, and JNKs, or p38 kinase (data not shown). Together with the results of the \textit{in vivo} phosphorylation assays, these data suggest that activation of p70S6K by UVA might require prior phosphorylation at Ser411 by JNKs and ERKs, Thr421/424 by ERKs or at other S/T-P sites possibly by p38 kinase and a second phosphorylation at Thr389 mediated by mTOR/PI-3 kinase or another unidentified kinase.

**p70S6K Proteins Co-immunoprecipitated with PI-3 Kinase or Possibly PDK1 are Activated by MAPKs In Vitro**—Prior phosphorylation of p70S6K at S/T-P sites by MAPKs was shown to be necessary for p70S6K activation through PDK1-mediated regulation, and the phosphorylation at Thr229 by PDK1 and at Thr389 by PI-3 kinase/mTOR was reported to engender partial activation of p70S6K, but total activation of p70S6K was not induced by MAPKs
alone (12, 22, 23, 28, 59). However, our present data showed that immunoprecipitated p70S6K proteins are activated by incubation with active MAPKs in vitro (Fig. 8B), whereas, the S6 peptide, a substrate of p70S6K, is not phosphorylated by active MAPKs in vitro (Fig. 8C). These results suggest that the p70S6K proteins may co-immunoprecipitate with other kinases that can phosphorylate the S6 peptide, or with additional p70S6K activating kinases such as PDK1. Our studies showed further that in the UVA-treated cells, the p70S6K proteins co-immunoprecipitated strongly with PI-3 kinase (Fig. 8D), very weakly with PDK1 (Fig. 8E), but not with Akt (Fig. 8F), as compared with corresponding controls. MAPK downstream kinases, including RSK1, RSK2, RSK3, or MSK1, can also phosphorylate the S6 peptide. However, the p70S6K immunoprecipitates were not contaminated with any phosphorylated or nonphosphorylated MAPK downstream kinases (data not shown). In addition, immunoprecipitates from normal non-immune serum did not contain any of the above-mentioned kinases (data not shown). Therefore, our results suggest that the activation and phosphorylation of p70S6K may be mediated by MAPKs in cooperation with PI-3 kinase and possibly PDK1 or an additional unidentified p70S6K-activating kinase.

Partial Reactivation of PP1-treated p70S6K Preparations by ERKs—PP1-treated p70S6K preparations were employed here to determine whether the IP-p70S6K used above was partially phosphorylated at non-MAPKs sites and, thus, be “primed” for its phosphorylation and activation by MAPKs in vitro. Our data showed the S6 kinase activity decreased by about 40% following treatment of the IP-p70S6K with PP1 compared to that with no PP1 treatment (Fig. 9A), indicating that a partial phosphorylation of IP-p70S6K exists in the unstimulated state. The partial phosphorylation appeared to facilitate induction of p70S6K activation by MAPKs (Figs. 8B and 9A). Furthermore, partial reactivation of PP1-treated IP-p70S6K was observed following
incubation with ERKs, p38 kinase, but not JNKs (Fig. 9A). Recently, a complex between ERKs and p70S6K was documented by immunoprecipitation (74). Therefore, these results suggest that p70S6K may be a potential substrate for ERKs or a kinase dependent upon ERKs, but full activation of p70S6K may occur via cooperation of MAPKs with PI3-kinase/PDK1 or an unidentified kinase.

DISCUSSION

Activation of p70S6K is known to occur through a hierarchical multi-site phosphorylation process directed at three separate domains: 1) a cluster of S/T-P sites (Ser411, Ser418, Thr421, Ser424) in an autoinhibitory domain in the noncatalytic C-terminal tail; 2) Thr229 in the activation loop of the catalytic domain; and 3) Thr389 and another two S/T-P sites (Ser371 and possibly Thr367) in the kinase extension domain immediately C-terminal to the catalytic domain (12, 22). Here, we employed phospho-specific anti-peptide antibodies to determine the changes in phosphorylation of p70S6K at Thr389, Ser411, and Thr421/Ser424 in response to UVA exposure. We found that exposure of JB6 cells to UVA irradiation stimulated p70S6K kinase activity in a time-dependent manner and simultaneously induced a dose- and time-dependent phosphorylation of p70S6K at the four sites, suggesting that phosphorylation at these sites may indirectly reflect p70S6K kinase activity. However, upstream kinase pathways leading to phosphorylation and activation of p70S6K are not well understood (12, 22, 25). Here, we used selective kinase inhibitors and specific dominant negative mutants of putative p70S6K upstream kinases to further analyze the roles of PI-3 kinase, mTOR and MAPKs in the regulation of UVA-stimulated p70S6K activation and phosphorylation.
Activation of the PI-3 kinase pathways was reported to be required for phosphorylation and activation of p70S6K (12, 23, 59). PDK1, a downstream kinase of PI-3 kinase, was clearly shown to be a markedly selective kinase for Thr229 phosphorylation contributing to p70S6K activation (12, 22, 25, 60). In addition, Thr389 phosphorylation was also shown to be mediated by PI-3 kinase/PDK1 and contributed to the activation of p70S6K (12, 22, 60). More recent studies indicated that Thr389 phosphorylation might create a PDK1 docking site in p70S6K that recruits and activates PDK1 leading to Thr229 phosphorylation and its activation (61). These previous studies suggest that phosphorylation at Th229 and Thr389 occurs through the PI-3 kinase pathway and reflects p70S6K activation in vivo. We observed that the changes in Thr389 phosphorylation correlate with those of p70S6K kinase activity when JB6 cells were exposed to UVA irradiation. Moreover, UVA-stimulated p70S6K activity and phosphorylation of Thr389, but not Ser411 or Thr421/Ser424, were prevented by wortmannin, a selective inhibitor of the PI-3 kinase p110 subunit, and also were abolished by a dominant negative mutant of the PI-3 kinase p85 subunit (DNM-Δp85), further suggesting that UVA-stimulated activation and phosphorylation at Thr389 may be regulated by the PI-3 kinase pathway but that the phosphorylation at the autoinhibitory domain S/T-P sites (Ser411, and Thr421/Ser424) occurs through PI-3 kinase independent pathways.

However, a kinase specific for Thr389 phosphorylation regulated by PI-3 kinase has not so far been identified. mTOR was shown to be a critical kinase in regulation of p70S6K activation. Immunoprecipitation studies showed that p70S6K at Thr389 was phosphorylated in vitro by mTOR (28, 71). But, other studies showed that regulation of p70S6K activation and Thr389 phosphorylation by mTOR occurs through inhibition of protein phosphatase 2A (PP2A)-mediated dephosphorylation (12, 22, 29, 31, 32). Here, UVA-stimulated p70S6K activity and
phosphorylation at Thr389 were completely abrogated by rapamycin, an mTOR inhibitor (20, 53), suggesting that mTOR is also required for p70S6K activation and phosphorylation at Thr389 induced by UVA exposure. On the other hand, UVA-induced phosphorylation at Ser411 and Thr421/Ser424 of p70S6K were only partially blocked by rapamycin. These data suggest that inhibition of phosphorylation of p70S6K at the four sites leading to its inactivation may occur via rapamycin-induced PP2A activation (12, 30, 51), inasmuch as insulin-induced inhibition of PP2A activity is blocked by rapamycin (62), and these rapamycin-sensitive S/T-P sites in the autoinhibitory domain do not serve as a substrate for mTOR in vitro (28). However, the sites in eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) phosphorylated by mTOR all contain an S/T-P motif (12), characteristic of sites phosphorylated by proline-directed kinases (e.g., MAPK). Therefore, mTOR might phosphorylate similar S/T-P motifs on p70S6K in the presence of an unidentified cofactor in vivo.

Although the role of MAPKs in the activation of the p70S6K signaling pathway has been controversial (12), phosphorylation of Thr229 by PDK1 and of Thr389 by mTOR in vitro results in only partial activation of p70S6K (12, 22, 25, 28), suggesting that complete activation of p70S6K may require additional pathways (e.g., MAPKs). Earlier studies employing dominant interfering mutants of Ras/Raf (35) as well as SH2 docking site mutants of platelet-derived growth factor (PDGF) (34) demonstrated that MAPKs are not necessary for p70S6K activation (35). However, later studies favor a role for autoinhibitory domain phosphorylation by MAPKs at the S/T-P sites for regulating p70S6K activation (23, 36). Thr229 phosphorylation required for p70S6K activation depends on prior phosphorylation of other sites that include the autoinhibitory domain S/T-P sites as well as Thr389 (28, 29, 63, 64). Recently, Weng et al. (22) hypothesized that phosphorylation of the S/T-P sites recognized by MAPKs is the first step in the hierarchical
multi-site phosphorylation of p70^{S6K} that leads to its activation. In our studies, UVA-stimulated p70^{S6K} activity and phosphorylation at Thr389 was inhibited by the MEK1 inhibitor, PD98059, and also markedly blocked by a dominant negative mutant of ERK2 (DNM-ERK2), suggesting that ERKs may be involved in p70^{S6K} activation and Thr389 phosphorylation. Furthermore, UVA-induced phosphorylation at Thr421/Ser424 and Ser411 was blocked by PD98059 and DNM-ERK2, consistent with other published reports (22, 23, 33, 36, 72). Therefore, our data and previous studies (22, 23, 25, 33, 36, 61, 64, 65) suggest that prior phosphorylation at Thr421/Ser424 and Ser411 by ERKs may induce a conformational change of p70^{S6K} that facilitates a second phosphorylation at Thr389 contributing to Thr229 phosphorylation and p70^{S6K} activation in response to UVA irradiation.

The p38 kinase and JNKs are putative upstream kinases that may be important in the regulation of p70^{S6K} activation (22). Wang, et al. (66) reported that p38 kinase but not JNKs is involved in activation of p70^{S6K} by arsenite. A p38 kinase inhibitor SB203580 prevented the activation of p70^{S6K} by mitogens (67). But recent experiments using the p38 kinase inhibitor, SB203580, and dominant mutants of p38 kinase and JNK pathways indicated that p38 kinase and JNKs are probably not implicated in the regulation of p70^{S6K} activity in response to osmotic stress (30). However, our studies suggest that p38 kinase may also be required for UVA-stimulated p70^{S6K} activation and phosphorylation at Thr389, inasmuch as UVA-stimulated p70^{S6K} activation and phosphorylation at Thr389 were blocked by a p38 kinase inhibitor, SB202190, and also inhibited by a dominant negative mutant of p38 kinase (DNM-p38). On the other hand, SB202190 and DNM-p38 had no effect on UVA-induced phosphorylation at Ser411 or Thr421/Ser424. These results suggest that prior phosphorylation of p70^{S6K} at S/T-P sites other than Ser411 and Thr421/Ser424 in the autoinhibitory domain may occur via activation of p38
kinase and facilitate a second phosphorylation at Thr389 and subsequent activation of p70^{S6K}. But another possibility that cannot be ruled out is that p38 kinase may be an activator of Akt (Nomura et al. and Liu et al., manuscripts in preparation) which might have an effect on p70^{S6K} activation.

Interestingly, PD169316, a novel inhibitor of p38 kinase and JNKs (57, 58), significantly blocked UVA-stimulated p70^{S6K} activity, consistent with the hypothesis that JNKs appear to be involved in p70^{S6K} activation (22, 68). Furthermore, UVA-induced p70^{S6K} activity and phosphorylation at Thr389 were significantly suppressed in JB6 cells expressing a dominant negative mutant of JNK1 (DNM-JNK1), and almost completely abrogated in Jnk1-/− and Jnk2-/− cells compared with their corresponding control cells, suggesting that activation of the JNKs pathway may be involved in UVA-stimulated p70^{S6K} activation. Moreover, UVA-stimulated phosphorylation of p70^{S6K} at Ser411 but not Thr421/Ser424 was inhibited in DNM-JNK1 cells, and also attenuated in Jnk1-/− and Jnk2-/− cells. These results indicated that prior phosphorylation at Ser411 but not Thr421/Ser424 may occur through activation of JNKs and facilitate a second phosphorylation at Thr389 and subsequent activation of p70^{S6K}.

To further examine the effects of MAPKs on phosphorylation and activation of p70^{S6K}, we performed in vitro experiments. Our data showed that the p70^{S6K} proteins immunoprecipitated from serum-free starved JB6 cell lysates were phosphorylated at Ser411 by active ERKs, and JNKs, but not p38 kinase, and at Thr421/Ser424 only by ERK1, agreeing at least partially with the results of Mukhopadhyay et al. (33). However, Thr389 phosphorylation was induced by MAPKs in vitro (data not shown). Together with the in vivo experiments, these data suggest that p70^{S6K} activation may be triggered through a prior phosphorylation at Ser411 by JNKs and ERKs, at Thr421/Ser421 by ERKs, or other S/T-P sites possibly by p38 kinase and
a second phosphorylation at Thr389 by a downstream unidentified kinase of PI-3 kinase/mTOR. Indeed, phosphorylation at the S/T-P sites in the autoinhibitory domain is confirmed to participate in phosphorylation of the p70S6K activation loop leading to its activation (25, 64, 65, 69). Additionally, we found that the p70S6K proteins precipitated with PI-3 kinase and possibly PDK1, but not with Akt or MAPK downstream kinases including RSK1, RSK2, RSK3, and MSK1 (Fig. 8 and data not shown). Furthermore, our data showed that the p70S6K complex possessed a partial basal level of phosphorylation and, thus, was “primed” for its activation by MAPKs in vitro, although p70S6K activity was shown not to be induced by MAPKs alone in vitro (12, 22, 23, 28, 59). In fact, active PI-3 kinase (p110) has been shown to induce p70S6K activation and phosphorylation at Thr229, Thr389, Ser411, and Thr421/Ser424 in vivo (22). Also, p70S6K forms a complex with PDK1 in co-transfected cells (70), but the binding site has not been identified. Recently, Gu et al. (74) reported that interrelationships between ERKs and p70S6K were characterized and a complex between both kinases was documented by immunoprecipitation, suggesting that p70S6K may be a potential substrate of ERKs or be dependent upon ERKs. This idea was supported by our experiments showing that PP1-deactivated p70S6K preparations were partially reactivated by ERKs in vitro, but the possibility of the existence of an unidentified kinase or a coactivator in our reaction system cannot be ruled out. Thus, these results suggest that full activation of p70S6K may be induced by cooperation of MAPKs with PI-3 kinase/PDK1 or an unidentified p70S6K-activating kinase. Another possibility remaining to be determined is that prior autoinhibitory domain phosphorylation by MAPKs may induce a conformational change in p70S6K that allows for a second phosphorylation at the S/T-P sites (Ser371 and possibly Thr367) in the kinase extension domain by MAPKs contributing to
p70\textsuperscript{S6K} activation (22, 26, 27). Overall, MAPKs, like PI-3 kinase/mTOR, may be required for phosphorylation and activation of p70\textsuperscript{S6K} by UVA.

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1 The abbreviations used are: UVA, UVB, or UVC, ultraviolet light A, B, or C; p70S6K, p70/p85 ribosomal S6 kinases; S/T-P, Proline-directed Ser/Thr; TPA, 12-O-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; PI, phosphatidylinositol; PIP3, phosphatidylinositol 3,4,5-triphosphate; PI-3 kinase, phosphatidylinositol-3 kinase; PDK1, 3-phosphoinositide-dependent protein kinase 1; mTOR, mammalian target of rapamycin; MAPKs, mitogen-activated protein kinases; ERKs, extracellular signal-regulated kinases; JNKs, c-jun N-terminal kinases; p38, p38 MAPK or p38 kinases; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; RSK, p90 ribosomal S6 kinases; MSK, mitogen- and stress-activated protein kinases; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; MEM, Eagle’s minimum essential medium; DMEM, modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; DTT, dithiothreitol; PKB, protein kinase B; PAGE, polyacrylamide gel electrophoresis; DNM, dominant negative mutant; p, phospho; np, nonphospho.
FIGURE LEGENDS

FIG. 1. **Activation and phosphorylation of p70^{S6K} at Ser411, Thr389, and Thr421/Ser424 induced by UVA.** (A) JB6 Cl 41 cells (8 x 10^5) were seeded into 100-mm dishes. After culturing for 24 h at 37 °C in humidified air with 5% CO_2, the cells were starved for 48 h by replacing medium with 0.1% FBS MEM. The medium was again replaced with fresh 0.1% FBS MEM and allowed to equilibrate for 4 h before treatment. Cells were untreated or exposed to UVA (160 kJ/m^2) or UVB (4 kJ/m^2). UVB-stimulated cell samples were used as a positive control and untreated samples were used as negative controls. After an additional incubation for 15, 30, or 60 min following UV treatment, the cells were lysed in buffer A for immunoprecipitation. The p70^{S6K} kinase activity was determined as described under “Experimental Procedures”. Each bar indicates the mean and standard deviation from four independent assays performed in duplicate. UVA-stimulated activity of p70^{S6K} was significantly higher than unstimulated control activity (* p<0.001). (B) JB6 Cl 41 cells were cultured as described above and then exposed to UVA, UVC, TPA, or EGF at the doses indicated. UVC-, TPA-, EGF-stimulated cell samples were used as positive controls and an untreated sample was used as a negative control. After an additional incubation for 30 min following the treatment, the cells were lysed in SDS sample buffer for immunoblotting. Phosphorylation of p70^{S6K} was determined as described under “Experimental Procedures”. The figure represents one of three similar independent experiments.

FIG. 2. **Dose- and time-dependent phosphorylation of p70^{S6K} at Ser411, Thr389, and Thr421/Ser424 induced by UVA.** JB6 Cl 41 cells (6 x 10^5) were cultured in 100-mm dishes until they reached 90% confluence and then were starved for 48 h in 0.1% FBS MEM. (A) The cells were irradiated with UVA at the doses indicated and harvested after 30 min and analyzed as
described under “Experimental Procedures”. (B) Cells were exposed to UVA (160 kJ/m²) and harvested at 15, 30, 120, 360, and 720 min following irradiation and analyzed as described under “Experimental Procedures”. This is one of three similar independent experiments and figures show that UVA-induced phosphorylation of 70S6K at Ser411, Thr389, and Thr421/Ser424 is dose- and time-dependent.

FIG. 3. Inhibition of activation and phosphorylation of p70S6K at Thr389 but not at Ser411 or Thr421/Ser424 by wortmannin and DNM-Δp85. JB6 Cl 41 or DNM-Δp85 cells (8 x 10⁵) were seeded into 100-mm dishes and cultured for 24 h in 5% FBS MEM. Then the cells were starved for 48 h in 0.1% FBS MEM. The Cl 41 cells were or were not pre-incubated for 1.5 h with wortmannin at the doses indicated and then irradiated with UVA (160 kJ/m²), whereas DNM-Δp85 cells were treated only with UVA at the doses indicated. The cell samples were harvested 15 or 30 min after irradiation. Phosphorylation of p70S6K and its kinase activity as well as PI-3 kinase activity were analyzed as described under “Experimental Procedures”. Some of the sample membranes were stripped and reprobed with different primary antibodies. This figure represents one of three independent similar experiments. The figures show that UVA-induced p70S6K phosphorylation at Thr389, but not at Ser411 or Thr421/Ser424, is blocked by wortmannin (A) and DNM-Δp85 (D). In addition, UVA-stimulated PI-3 kinase activity was abolished by wortmannin (B) and DNM-Δp85 (E). Cells were harvested 15 min (B and E) or 30 min (A and D) or at the time indicated (C and F) after UVA irradiation. Each bar represents the mean and standard deviation from three independent assays performed in duplicate. UVA (160 kJ/m²)-induced p70S6K activity was significantly inhibited (**, p<0.001) by wortmannin (0.2 μM) (C) or DNM-Δp85 (F) compared to corresponding positive controls.
FIG. 4. **Inhibition of UVA-induced activation and phosphorylation of p70S6K at Thr389, Ser411, and Thr421/Ser424 with rapamycin.**  Cl 41 cells (8 x 10^5) were cultured in monolayers for 24 h in 100-mm dishes and subsequently starved for 48 h in 0.1% FBS MEM. The cells were pretreated for 1.5 h with rapamycin at the doses indicated. Then the cells were harvested 15 or 30 min after irradiation with UVA (160 kJ/m^2). The phosphorylation of p70S6K proteins and S6 kinase activity were determined as described under “Experimental Procedures”. The sample membrane was stripped and reprobed with different antibodies. (A) Shows that rapamycin inhibits phosphorylation of p70S6K at Thr389, Ser411, and Thr421/Ser424 (30 min). This is one of three similar independent experiments. (B) Each bar indicates the mean and standard deviation from three independent assays performed in duplicate. UVA-induced p70S6K activity was significantly inhibited (*, p<0.01) by rapamycin (100 mM) compared to corresponding positive controls.

FIG. 5. **Inhibition of UVA-induced activation and phosphorylation of p70S6K at all four sites by PD98059 or DNM-ERK2.**  JB6 Cl 41 or DNM-ERK2 cells were cultured for 24 h in each well of a 6-well plate until 90% confluence was reached. The cells were starved for 48 h in 0.1% FBS MEM and then harvested 15 or 30 min after UVA irradiation or a combination of UVA and pretreatment with PD98059 at the doses indicated. Total and phosphorylated p70S6K, ERKs, and p38 kinase, as well as S6 kinase activity, were determined as described under “Experimental Procedures”. The sample membrane was stripped and reprobed with different antibodies. This is one of three similar independent experiments. (A) The figure shows that PD98059 blocks UVA-induced phosphorylation of ERKs and p70S6K at Thr389, Ser411, and...
Thr421/Ser424 (30 min). (B) Shows that DNM-ERK2 suppresses phosphorylation of ERKs and p70S6K at Thr389/Ser411 and Thr421/Ser424 (harvested at 30 min). (D) Each bar represents the mean and standard deviation from three independent assays performed in duplicate. UVA (160 kJ/m²)-induced p70S6K activity was significantly inhibited (**, p<0.001) by PD98059 (25 µM) (C) or DNM-ERK2 (D) compared to corresponding positive controls.

FIG. 6. Inhibition of UVA-induced activation and phosphorylation of p70S6K at Thr389 but not Ser411 or Thr421/Ser424 by DNM-p38 and SB202190. JB6 Cl 41 or DNM-p38 cells were treated as described in Fig. 5. The cells were irradiated with UVA either following SB202190 pretreatment or with no pretreatment. Total and phosphorylated p70S6K, p38 kinase, and ERKs, as well as p70S6K S6 kinase activity, were determined as described under “Experimental Procedures”. The sample membrane was stripped and reprobed with different antibodies. This is one of three similar independent experiments. (A) The figure shows that SB202190 inhibits UVA-induced phosphorylation of p38 kinase and p70S6K at Thr389, but not Ser411 or Thr421/Ser424 (harvested at 30 min). (B) DNM-p38 also blocks phosphorylation of p38 kinase and p70S6K at Thr389, but not at Ser411 or Thr421/Ser424 (30 min). Each bar indicates the mean and standard deviation from three independent assays performed in duplicate. UVA (160 kJ/m²)-induced p70S6K activity was significantly inhibited by SB202190 (1.0 µM), PD169316 (0.5 µM) (C) or DNM-p38 (D) compared to corresponding positive controls (*, p<0.05; **, p<0.001).

FIG. 7. Inhibition of UVA-induced activation and phosphorylation of p70S6K at Thr389 and Ser411, but not at Thr421/Ser424 in DNM-JNK1 and Jnk1-/Jnk2-/ cells. JB6 Cl 41 or DNM-JNK1 cells were treated as described for Fig. 5. Preparation and treatment of primary
embryo fibroblasts of wild-type Jnk+/+ and Jnk1/- or Jnk2/- knockout mice and analysis of p70S6K and JNKs and their phosphorylated proteins as well as of p70S6K kinase activity, were performed as described under “Experimental Procedures”. The sample membrane was stripped and reprobed with different antibodies. This is one of three similar independent experiments. The figures show that DNM-JNK1 inhibits UVA-induced phosphorylation of JNKs and p70S6K at Thr389 and Ser411, but not Thr421/Ser424 (A), and also partially suppresses p70S6K kinase activity following exposure to UVA (160 kJ/m²) (C). Moreover, phosphorylation of JNKs and p70S6K at Thr389 and Ser411, but not Thr421/Ser424 (B), as well as p70S6K kinase activity induced by UVA (80 kJ/m²) (D), were also almost completely attenuated in Jnk1/- and Jnk2/- cells. Each bar represents the mean and standard deviation from three independent experiments performed in duplicate. UVA-induced p70S6K activity in DNM-JNK1, Jnk1/- or Jnk2/- cells was significantly inhibited compared to corresponding positive controls (*, p<0.05; **, p<0.001).

FIG. 8. Co-immunoprecipitates of p70S6K and PI-3 kinase or possibly PDK1 are phosphorylated and activated by active MAPKs in vitro. After starvation of JB6 cells for 48 h, the cell lysates were subjected to immunoprecipitation (IP) with rabbit anti-p70S6K polyclonal antibody. (A) The immune-complexes were incubated with or without MAPKs including ERK1, ERK2, JNK1, JNK2, or p38 kinase (Upstate Biotechnology Inc.) in the amounts indicated. Then the reactions were analyzed by western immunoblotting with mouse anti-phospho-specific p70S6K monoclonal antibody (Ser411) or rabbit anti-phospho-specific p70S6K polyclonal antibodies (Thr421/Ser424). The figure is one of three similar independent experiments. (B) The IP-p70S6K proteins were incubated in vitro in kinase buffer with MAPKs and the S6 peptide as a
substrate of p70<sup>S6K</sup>. Addition of BSA instead of MAPKs was used as an internal control. (C) the S6 peptide was incubated with or without MAPKs in kinase buffer containing Protein A/G plus Sepharose. (B) Each bar represents the mean and standard deviation from three independent assays performed in duplicate. Immunoprecipitates of p70<sup>S6K</sup> were significantly activated (*, p<0.05; **, p<0.01) in the presence of the different MAPKs versus the control containing no MAPK or the control containing no immunoprecipitated p70<sup>S6K</sup> (p<0.0001). (D) IP- p70<sup>S6K</sup> and IP-PI-3 kinase were precipitated from UVA-irradiated or nonirradiated cell lysates and then subjected to the PI-3 kinase assay as described under “Experimental Procedures”. This is one of three similar independent experiments. (E and F) p70<sup>S6K</sup>, IP-PDK1 and IP-Akt from UVA-irradiated or nonirradiated cell lysates were precipitated and then subjected to western immunoblotting analysis with anti-PDK1, -Akt or phospho-Akt (Ser473) antibodies, respectively. The figure is one of three similar independent experiments. IP: immunoprecipitate.

FIG. 9. Partial reactivation of PP1-dephosphorylated IP-p70<sup>S6K</sup> preparations by ERKs. (A) The p70<sup>S6K</sup> preparations from serum-starved JB6 Cl 41 cells were immunoprecipitated with p70<sup>S6K</sup> antibody and then dephosphorylated by PP1 (0.5 unit) treatment (72, 73). Then, the PP1 activity was inhibited by the addition of NaF (10 mM). Subsequently, PP1-deactivated p70<sup>S6K</sup> preparations were incubated with ERK1 (10 ng/µl), ERK2 (10 ng/µl), p38 kinase (10 ng/µl), JNK1 (50 mU/µl), or JNK2 (50 mU/µl) or without MAPKs (as a control) and S6 kinase activity assay was performed as described under “Experimental Procedures”. Each bar indicates the mean and standard deviation from two independent experiments performed in duplicate. Partial reactivation of PP1-treated p70<sup>S6K</sup> by ERKs is different (*, p<0.05) from that of corresponding control by no MAPKs. (B) Shows concise procedures of the above-mentioned experiments.
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Fig. 1

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative p70S6K activity (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>1.0</td>
</tr>
<tr>
<td>UVA 15 min</td>
<td>* (3.5)</td>
</tr>
<tr>
<td>UVA 30 min</td>
<td>* (4.5)</td>
</tr>
<tr>
<td>UVA 60 min</td>
<td>* (4.0)</td>
</tr>
<tr>
<td>UVB</td>
<td></td>
</tr>
</tbody>
</table>

B

- p-p85S6K
- p-p70S6K(Ser411)
- p-p85S6K
- p-p70S6K(Thr421/Ser424)
- p-p85S6K
- p-p70S6K(Thr389)
- np-p70S6K
Fig. 2

A

- 2 40 80 160 UVA (kJ/m²)

- p-p85S6K
- p-p70S6K(Ser411)
- p-p85S6K
- p-p70S6K(Thr421/Ser424)
- p-p70S6K(Thr389)
- np-p70S6K

B

15 30 120 360 720 min, after UVA

UVA (160 kJ/m²)

- p-p85S6K
- p-p70S6K(Ser411)
- p-p70S6K(Thr421/Ser424)
- p-p85S6K
- p-p70S6K(Thr389)
- np-p70S6K
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Fig. 3

A. Western blots showing the expression of p-p70S6K(Ser411), p-p85S6K, p-p70S6K(Thr389), p-p70S6K(Thr421/Ser424), np-p70S6K in cells treated with various concentrations of wortmannin and UVA radiation.

B. Western blots showing the expression of PIP3 and Ori in cells treated with wortmannin and UVA radiation.

C. Graph showing the relative p70S6K activity (fold) in control, UVA, and UVA+Wortmannin-treated cells at 15 and 30 min. The graph indicates significant increases in p70S6K activity with UVA and UVA+Wortmannin treatments, with the latter showing a more pronounced effect.

D. Western blots showing the expression of p-p70S6K(Ser411), p-p85S6K, p-p70S6K(Thr389), p-p70S6K(Thr421/Ser424), np-p70S6K in cells treated with different concentrations of UVA radiation.

E. Western blots showing the expression of PIP3 and Ori in cells treated with different concentrations of UVA radiation.

F. Graph showing the relative p70S6K activity (fold) in cells treated with UVA radiation for 15 and 30 min. The graph shows significant increases in p70S6K activity with UVA treatment, with a more pronounced effect at 30 min.
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Fig. 4

Graph A shows the effects of rapamycin and UVA on p-p70S6K (Ser411), p-p70S6K (Thr421/Ser424), p-p70S6K (Thr389), and np-p70S6K in different treatments.

Graph B displays the relative p70S6K activity (fold) over 15 and 30 minutes, comparing Control, UVA, and UVA + rapamycin treatments.
Fig. 5

A

- - 6.25 12.5 25 50

PD98059 (µM)

UVA 160 kJ/m²

- + + + + +

p-p70S6K(Ser411)

-p-p70S6K(Thr421/Ser424)

-p-p85S6K

-p-p70S6K(Thr389)

-np-p70S6K

-p-ERKs

-np-ERKs

p-p38 kinase

B

Cl 41 DNM-ERK2

- 40 80 160

UVA (kJ/m²)

- 40 80 160

p-p70S6K(Ser411)

-p-p70S6K(Thr421/Ser424)

-p-p85S6K

-p-p70S6K(Thr389)

-np-p70S6K

-p-ERKs

-np-ERKs

p-p38 kinase

C

Relative p70S6K activity (fold)

15 min 30 min

Control UVA UVA+PD98059

D

Relative p70S6K activity (fold)

Cl 41 DNM-ERK2

Ctrl UVA 15 min UVA 30 min
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Fig. 6

A

- - 0.25 0.5 1.0 2.0 SB202190 (µM)
UVA 160 kJ/m²
-p-p70S6K(Ser411)
-p-p70S6K(Thr421/Ser424)
-p-p70S6K(Thr389)
-np-p70S6K
-p-p38 kinase
-np-p38 kinase
-p-ERKs

B

Cl 41
DNM-p38

UVA (kJ/m²)
- 40 80 160 - 40 80 160
-p-p70S6K(Ser411)
-p-p70S6K(Thr421/Ser424)
-p-p85S6K
-p-p70S6K(Thr389)
-np-p70S6K
-p-p38 kinase
-np-p38 kinase
-p-ERKs

C

Relative p70S6K activity (fold)
- 0 1 2 3 4 5 6 7 8
15 min 30 min

D

Relative p70S6K activity (fold)
- 0 1 2 3 4 5 6 7 8
Ctrl UVA 15 min UVA 30 min
Fig. 7

**Panel A**
Cl 41 and DNM-JNK1 were treated with different concentrations of UVA (kJ/m²). Western blot analysis was performed to detect the phosphorylation of p70S6K (Thr389), p70S6K (Thr421/Ser424), and p85S6K.

**Panel B**
Phosphorylation of p70S6K was compared in Jnk+/+, Jnk1−/−, and Jnk2−/− cells treated with UVA (kJ/m²).

**Panel C**
Bar graph showing the relative p70S6K activity (fold) in Ctrl, UVA 15 min, and UVA 30 min treated cells.

**Panel D**
Relative p70S6K activity (fold) in Jnk+/+, Jnk1−/−, and Jnk2−/− cells treated with different UVA exposure times.
Fig. 8

- **A**
  - Western blot analysis showing p-p70S6K (Ser411) and p-p70S6K (Thr421/Ser424) with IP-p70S6K.

- **B**
  - Graph showing relative p70S6K activity (fold) with error bars. Significance levels indicated by ** and *.

- **C**
  - Graph showing S6 phosphorylation (fold) with error bars.

- **D**
  - Autoradiogram showing IP-p70S6K and IP-PI3-Kinase activity with time points (min, after UVA).

- **E**
  - Autoradiogram showing IP-p70S6K and IP-PDK1 with time points (min, after UVA).

- **F**
  - Autoradiogram showing IP-p70S6K and IP-Akt with time points (min, after UVA).
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Fig. 9

**A**

![Graph showing relative p70S6K activity](image)

**B**

- Serum-starved cell lysates
- IP-p70S6k preparation
- Dephosphorylation with PP1
- Inhibition of PP1 with NaF
- Re-phosphorylation by MAPKs
- S6 kinase activity assay
Signal transduction pathways involved in phosphorylation and activation of p70S6K following exposure to UVA irradiation
Yiguo Zhang, Ziming Dong, Masaaki Nomura, Shuping Zhong, Nanyue Chen, Ann M. Bode and Zigang Dong

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