UVA exposure plays an important role in the etiology of skin cancer. The family of p90-kDa ribosomal S6 kinases (p90RSK/MAPKAP-K1) are activated via phosphorylation. In this study, results show that UVA-induced phosphorylation of p90RSK at Ser^{381} through ERKs and JNKs, but not p38 kinase pathways. We provide evidence that UVA-induced p90RSK phosphorylation and kinase activity were time- and dose-dependent. Both PD98059 and a dominant negative mutant of ERK2 blocked ERKs and p90RSK Ser^{381} phosphorylation, as well as p90RSK activity. A dominant negative mutant of p38 kinase blocked UVA-induced phosphorylation of p38 kinase, but had no effect on UVA-induced Ser^{381} phosphorylation of p90RSK or kinase activity. UVA-induced p90RSK phosphorylation and kinase activity were markedly attenuated in JNK^{-/-} and JNK2^{-/-} cells. A dominant negative mutant of JNK, inhibited UVA-induced JNKs and p90RSK phosphorylation and kinase activity, but had no effect on ERKs phosphorylation. PD169316, a novel inhibitor of JNKs and p38 kinase, inhibited phosphorylation of p90RSK. JNKs, and p38 kinase, but not ERKs. However, SB202190, a selective inhibitor of p38 kinase, had no effect on p90RSK or JNKs phosphorylation. Significantly, ERKs and JNKs, but not p38 kinase, immunoprecipitated with p90RSK when stimulated by UVA and p90RSK was a substrate for ERK2 and JNK2, but not p38 kinase. These data indicate clearly that p90RSK Ser^{381} may be phosphorylated by activation of JNKs or ERKs, but not p38 kinase.

The incidence of nonmelanoma and melanoma skin cancers has been increasing for several decades in most parts of the world (1–3), but mainly in populations of European origin (3, 4). Approximately 90% of nonmelanoma skin cancers are thought to be caused by ultraviolet (UV) exposure (5–7). The UV part of the solar electromagnetic spectrum is divided into UVC (200–290 nm), UVB (290–320 nm), and UVA (320–400 nm) (5, 8), and UVA is also subdivided into UVA2 (320–340 nm) and UVA1 (340–400 nm) (9, 10). Although UVC radiation can induce skin cancers by damaging DNA (11), UVC is not pertinent to sunlight-induced human carcinogenesis because UVC is completely absorbed by the earth’s stratospheric ozone layer and does not reach the surface of the earth (9). On the other hand, although UVB is only absorbed partially by the ozone layer (5, 6, 9) and can induce skin cancer by generating DNA damage (11), the proportion of UVB in the solar UV is small. Therefore, the risk of UVB-induced acute and chronic damage, including skin cancer (4, 8, 11), is diminished and can be blocked by limiting sunlight exposure during midday hours, wearing protective clothing, and using sunscreens (4, 8). On the other hand, UVA is not absorbed by the ozone layer and thus the amount of UVA radiation reaching the earth’s surface is ~20 times greater than that of UVB (5, 8). Therefore, UVA may be a major component of the solar UV radiation contributing to skin cancer. Results from epidemiological (4, 8) and animal (5, 6, 8, 12) studies support the concept that recreational UV exposure may play an important role in the etiology of human skin cancer. UVA-induced signal transduction pathways may be a significant component in the mechanism of UV-induced carcinogenesis (5, 6, 9, 13). However, most recent reports focus on UVC- or UVB-induced signal transduction (13, 14) and little is known regarding pathways induced by UVA.

Extracellular signals have been shown to activate mitogen-activated protein kinase (MAPK) cascades including extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), and p38 kinase (p38) (15). Among the first substrates of ERKs are the family of 90-kDa ribosomal S6 kinases (p90RSK), also known as MAPKAP-K1 (16). The MAPKAP-K1 family is activated via phosphorylation and shown to be ubiquitous and versatile mediators of signal transduction (17, 18). These signaling molecules are of much interest due to their potent ability to be phosphorylated by activation of ERKs (18) and their involvement in regulation of various cellular functions (16, 19). Initially, p90RSK was isolated from Xenopus and identified on the basis of its ability to phosphorylate the 31-kDa protein S6 that is a component of the 40 S ribosomal subunit in vitro (20, 21). As a signal-transducing serine/threonine kinase, p90RSK is an important member of a growing subfamily of MAPKs-activated protein kinases (16, 18, 22) that contain two distinct kinase catalytic domains in a single polypeptide chain. The two domains are the amino-terminal kinase domain (NTD) and the carboxyl-terminal kinase domain (CTD) (16, 22). With regard to primary structure,
the NTD of p90RSK is most closely related to p70S6 kinase (p70S6K) (16, 23). It was shown to phosphorylate exogenous substrates for p90RSK, including the cAMP response element-binding protein (24), c-Fos (25), and the estrogen receptor (26). These substrates suggest that p90RSK may play a role in transcriptional regulation. The CTD of p90RSK is related to a camodulin-dependent protein kinase and is most similar to phosphorylase kinase (16). The NTD may also be activated by phosphorylation of the CTD (16). ERKs were shown to phosphorylate and activate p90RSK in vivo (27, 29). To date, six phosphorylation sites have been identified, three of which are phosphorylated by MAPks in vitro (28). MAPks-catalyzed phosphorylation of Ser\textsuperscript{381} and Thr\textsuperscript{384} is critical for activation of the NTD and CTD, respectively, and the phosphorylation of Ser\textsuperscript{381} catalyzed by the CTD is also important for activation of the NTD (28, 29). The Ser\textsuperscript{381} site is located on a linker region between NTD and CTD (16, 30). Recent studies suggest that phosphorylation of Ser\textsuperscript{381} creates a docking site for PDK1 (31).

In mammalian cells, p90RSK is activated in response to a broad range of cellular perturbations (16, 32, 33), including oncogenic transformation (32), insulin (17, 32), growth factors (16, 32), JNKs (Thr\textsuperscript{183}/Tyr\textsuperscript{185}) and p38 kinase (Thr\textsuperscript{180}/Tyr\textsuperscript{182}), and anti-apoptotic death (16). In this study, we demonstrated that ERKs and JNKs, but not p38 kinase, are involved in UVA-induced p90RSK activation and subsequently transferred onto immunoprecipitated p90RSK from Western immunoblotting using a chemiluminescent detection system (ECL, New England Biolabs, Inc.). Some transfer membranes were washed with stripping buffer (7 mM guanidine hydrochloride, 50 mM glycine, pH 10.8, 0.05 mM EDTA, 0.1 mM KCl, and 20 mM β-mercaptoethanol) and reprobed with other primary phospho-specific or nonphospho-specific antibodies.

**Analysis of p90RSK Phosphorylation with Phospho-specific Antibodies—**Cells (5 × 10\textsuperscript{6}) were seeded into 100-mm dishes and cultured for 24 to 48 h. After the cells reached 80-90% confluence, the CI 41, DNM-ERK2, DNM-JNK1, or DNM-p38 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 UVA irradiation), the cells were washed once with ice-cold phosphate-buffered saline and lysed in 200 μl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue). The lysed samples were assayed for 30 min at 37 °C. 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 mM guanidine hydrochloride, 50 mM glycine, pH 10.8, 0.05 mM EDTA, 0.1 mM KCl, and 20 mM β-mercaptoethanol) and reprobed with other primary phospho-specific or nonphospho-specific antibodies.

**UVA Irradiation of Cells—**The UVA source used was a Philips TL100w/10R system from Ultraviolet Resources International (Lake-wood, 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 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 also produce a small amount of UVB and UVC.

**Stable Transfectants and Cell Culture—**The CMV-neo vector plasmid was constructed as previously reported (34). Mouse epidermal JB6 primary keratinocytes were used to stably transfect the DNA of the CTD (CI 41) (35) or with dominant negative mutant cell lines for JNK1 (DNM-JNK1) (34), p38 kinase (DNM-p38) (36), or ERK2 (DNM-ERK2) (37) as described above (35–37). They were 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 a humidified air with 5% CO\textsubscript{2}. Before each experiment, transfectants were selected with G418 and tested with their phospho-specific MAPK antibodies.

**Phosphorylation of ERKs, JNKs, and p38—**Immunoblot analysis for phosphorylated proteins of ERKs, JNKs, and p38 kinase was carried out using the phospho-specific MAPK antibodies as reported previously (34–37). STAT3 was used as an internal control to determine equal loading of protein. 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 mM guanidine hydrochloride, 50 mM glycine, pH 10.8, 0.05 mM EDTA, 0.1 mM KCl, and 20 mM β-mercaptoethanol) and reprobed with other primary phospho-specific or nonphospho-specific antibodies.
UVA Induces p90RSK Phosphorylation via ERKs and JNKs

Co-immunoprecipitation of Proteins—Jb6 Cl 41 cell lysates were prepared as described above. Following exposure to UVA (160 kJ/m²), cells were harvested at 15 or 30 min and lysed for 5 min in buffer A. Supernatant fractions were incubated at 4 °C overnight with normal rabbit serum as a non-immune IgG control or with antibodies against ERKs, JNKs, p38 kinase, or p90RSK (MAPKAP-K1a) and for an additional 4 h with protein-A/G plus Sepharose (4 °C). Samples were then washed four times with phosphate-buffered saline and the final pellet resuspended in 3 × SDS sample buffer. The immunoprecipitated proteins were analyzed by using Western immunoblotting as recommended by New England BioLabs, Inc. (40, 41). Immunoprecipitates of ERKs, JNKs, or p38 kinase were incubated with anti-phospho-p90RSK (Ser 381) antibody (Upstate Biotechnology, Inc.) and p90RSK (MAPKAP-K1a) were incubated with phospho-specific ERKs, JNKs, or p38 kinase as the primary antibody.

Preparation and Analysis JnK 

Primary Embryo Fibroblasts—Embryo fibroblasts from normal JnK 

embryo fibroblasts from normal JnK 

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 penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. For analysis of protein phosphorylation and kinase activity, the cells were starved by replacing growth medium with serum-free DMEM for 24 h at which time they were exposed to UVA. The cells were lysed with SDS sample buffer and protein concentration in the cell lysates was determined (Bio-Rad assay). Equal amounts of protein were loaded onto an 8% SDS-polyacrylamide gel and phosphorylated and nonphosphorylated proteins were determined by Western blotting analysis. Kinase activity of p90RSK in these cells was performed as described above.

In Vitro Assay for p90RSK Phosphorylation and Activation (29, 43)—Jb6 Cl 41 cell lysates were cultured in 100-mm dishes and starved for 24 h and co-immunoprecipitation experiments with p90RSK (MAPKAP-K1a) were performed as described above. Samples containing immunoprecipitated p90RSK (MAPKAP-K1a) were incubated at 30 °C for 60 min with active ERK2, JNK2, or p38 kinase (10 ng/ml) (Upstate Biotechnology, Inc.) in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 1 mM diithiothreitol, 5 mM ATP, and 0.01% Brij 35). At the same time, kinase activity of ERK2, JNK2, or p38 kinase was tested by incubating with kinase substrates, Elk1, or ATF2 fusion proteins (1 mg/ml) (New England BioLabs, Inc.). Reactions were stopped by adding SDS sample buffer. The phosphorylation of kinase protein substrates and immunoprecipitated p90RSK protein was analyzed using SDS-polyacrylamide gel electrophoresis, Western blotting, and a chemiluminescent detection system (Amer sham Pharmacia Biotech, Inc.). Total lysates taken directly from Cl 41 cells that were irradiated with UVA (160 kJ/m²) were used as an internal control. To further analyze whether p90RSK is activated by MAPKs in vitro, samples containing immunoprecipitated p90RSK were incubated at 30 °C for 30 min with S6 peptide plus active ERK1, ERK2, JNK1, JNK2, or p38 kinase (10 ng/ml, Upstate Biotechnology, Inc.) and p90RSK kinase activity was determined as described above. At the same time, incubations of S6 peptide with MAPKs were used as internal controls.

Data Analysis—Some data were analyzed using the Image-QuaNT™ Microsoft System (Molecular Dynamics, Sunnyvale, CA). This system calculates the intensity of bands in Western blots.

Statistical Analysis—Significant differences in p90RSK S6 kinase activity were determined by using Student’s t test.

RESULTS

Phosphorylation of p90RSK at Ser 381 Is Induced by UVA, TPA, and EGF—TPA is a known tumor promoter and acts during tumor promotion and progression (44, 45). EGF is a well described growth factor having tumor promoting action (45). TPA and EGF were reported to activate p90RSK via phosphorylation (16, 17, 39, 40) and were used here as positive controls for comparison. The phosphorylation of p90RSK was measured using specific antibodies against p90RSK. Our results show that like TPA and EGF, UVA induced the phosphorylation of p90RSK (Fig. 1A). In contrast to the non-irradiated control, UVA at a higher dose (160 kJ/m²) induced a 4.7-fold increase in phosphorylation of p90RSK, which was 2.8 times that of either TPA or EGF (Fig. 1B).

UVA-induced Ser 381 Phosphorylation of p90RSK Is Dose- and Time-dependent—As shown in Figs. 1 and 2A, UVA-induced phosphorylation of p90RSK was dose- and time-dependent.

FIG. 1. Dose-dependent phosphorylation of p90RSK Ser 381 induced by UVA. Jb6 Cl 41 cells (5 × 10⁶) were seeded into 100-mm dishes. After culturing for 24 h at 37 °C in humidified air with 5% CO₂, the cells were starved for 48 h by replacing media with 0.1% FBS/MEM. The media was again replaced with fresh 0.1% FBS/MEM 4 h before exposure to UV irradiation or chemicals. The cells were exposed to UVA, TPA, or EGF at doses indicated. TPA- or EGF-stimulated cell samples were used as positive controls. After an additional incubation of 30 min, the treated cells were lysed in SDS sample buffer and p90RSK protein and its phosphorylated protein (A) were determined as described under “Experimental Procedures.” For comparison, the lower panel (B) shows the ratio of phosphorylation in each stimulated sample to unstimulated control (value of 1) using the Image-QuaNT™ Microsoft System. B, bars, correspond directly to bands in A. B, data are presented as the mean and standard deviation of three independent assays and the change in UVA-, TPA-, or EGF-stimulated phosphorylation is significantly different from the unstimulated control (*, p < 0.05; **, p < 0.01).

PD98059 Inhibits UVA-induced Ser 381 Phosphorylation of p90RSK—Coinciding with Inhibition of ERKs Phosphorylation—PD98059, an inhibitor for MAP kinase kinase (MEK1) (40, 46), was used in experiments to study the role of MEK1/ERKs in the UVA-induced Ser 381 phosphorylation of p90RSK. PD98059 markedly inhibited both UVA-induced phosphorylation of ERKs and p90RSK at Ser 381 (Fig. 3, A and B). At the same time, PD98059 significantly blocked p90RSK S6 kinase activation by UVA (Fig. 3E). In contrast, PD98059 was less effective in inhibiting UVA-induced phosphorylation of JNKs and p38 kinase (Fig. 3, C and D). Additionally, p90RSK S6 kinase activation was also blocked by LY294002 (Fig. 3E), a PI 3-kinase inhibitor, which was used here as a positive control. These data suggest that Ser 381 phosphorylation and activation of p90RSK may be dependent on ERKs.

DNN-ERK2 Blocks UVA-induced Phosphorylation of Both
p90\textsuperscript{RSK} at Ser\textsuperscript{381} and ERKs—The concept that PD98059 is a MEK1-specific inhibitor was challenged by the report of Kadowaki et al. (47), in which PD98059 was shown to inhibit ERK2 activity when the cells were stimulated by UVA (Fig. 5, A and B), but inhibited phosphorylation of p38 kinase induced by UVA (Fig. 5B). PD98059 also had no significant inhibitory effect on p90\textsuperscript{RSK} kinase activity when the cells were stimulated by UVA (Fig. 5, A and B). These data suggested that the Ser\textsuperscript{381} site in p90\textsuperscript{RSK} is phosphorylated via the ERKs pathway, but not through the p38 kinase pathway. Additionally, the basal expression levels of p90\textsuperscript{RSK} at Ser\textsuperscript{381} and Thr\textsuperscript{360/Ser\textsuperscript{364}} (Fig. 6B) were lower in DNM-ERKs cells than those in control JnK\textsuperscript{−/−} cells. Our results showed that basal and phosphorylated levels of p90\textsuperscript{RSK} at Ser\textsuperscript{381} and Thr\textsuperscript{360/Ser\textsuperscript{364}} (Fig. 6A) and JNKs (Fig. 6B), as well as p90\textsuperscript{RSK} kinase activity (Fig. 6D), were lower in JnK\textsuperscript{−/−} and JnK\textsuperscript{−/−} cells than those in control JnK\textsuperscript{−/+} cells. However, basal and phosphorylated ERKs (Fig. 6C) were not significantly changed in JnK\textsuperscript{−/−} and JnK\textsuperscript{−/−} cells.
DNM-JNK1 Inhibits UVA-induced Phosphorylation of p90^RSK and JNKs—To further confirm that UVA-induced phosphorylation of p90^RSK occurs through the JNK pathway in vivo, a dominant negative mutant of JNK1 (34) was used in this experiment. Our results showed that UVA-induced phosphorylation of p90^RSK at Ser^{381} (Fig. 7, A and D) and JNKs (Fig. 7B), as well as p90^RSK S6 kinase activity (Fig. 7E), were inhibited in DNM-JNK1 cells, but inhibition of ERKs phosphorylation was not observed (Fig. 7C). These data indicated that the JNK pathway may play a role in UVA-induced phosphorylation and activation of p90^RSK.

PD169316 but Not SB202190 Blocks UVA-induced Ser^{381} Phosphorylation and Activation of p90^RSK—The findings above (Fig. 5) suggest that phosphorylation and activation of p90^RSK appear to occur through a p38 kinase independent pathway. This idea was further supported by evidence showing that, SB202190, a selective inhibitor of p38 kinase (48), only inhibited phosphorylation of p38 kinase, but not phosphorylation of JNKs, ERKs (Fig. 8B), or p90^RSK at Ser^{381} (Fig. 8A). Another compound, PD169316, is confirmed to be a novel inhibitor of JNKs and p38 kinase (49, 50) and it completely blocked UVA-induced phosphorylation of JNKs, p38 kinase (Fig. 8B), and p90^RSK Ser^{381} (Fig. 8A), but not that of ERKs (Fig. 8B). Our data also showed that p80^RSK S6 kinase activity was blocked markedly by PD169316, but not by SB202190 (Fig. 8C). These data further indicate that UVA-induced phosphorylation and activation of p90^RSK are mediated through the JNKs, but not the p38 kinase pathway.

p90^RSK Co-immunoprecipitates with ERKs or JNKs, but Not p38 Kinase—The non-phosphorylated-p90^RSK protein-antibody complex was co-immunoprecipitated strongly with anti-phospho-ERKs and weakly with anti-phospho-JNKs, but not at all with anti-phospho-p38 kinase as determined by Western immunoblotting analysis (Fig. 9A). Inversely, a UVA-induced phosphorylated p90^RSK was co-immunoprecipitated weakly with the immunoprecipitates containing nonphospho-ERKs (Fig. 9B) and -JNKs (Fig. 9C) antibodies, but not with those containing nonphospho-p38 kinase antibody (Fig. 9D). At the same time, immunoprecipitates with normal non-immune IgG
D, ERKs was not significantly inhibited in the two knockout cell lines (C1/JNK of MAP kinases in the phosphorylation of p90RSK, whereas JNK2, but not p38 kinase in vitro.

To further study the role but not p38 kinase. Whereas the ERK-docking site in p90RSK phosphorylated by JNK2 and p38 kinase (Fig. 10, B). The phosphorylated p90RSK, JNKs, and ERKs proteins, as well as p90RSK. S6 kinase activity and phosphorylation at Ser381 and Thr362/Ser381, as well as JNKs and ERKs and their phosphorylated proteins, were performed as described above.

**DISCUSSION**

Two major signaling pathways regulating cell function that are activated by various stimuli (16, 52, 53, 54) include: 1) the phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase A kinase activity and phosphorylation at Ser381 and Thr362/Ser381. As shown in Fig. 10, Elk1, a substrate was for ERK2, phosphorylated by ERK2 (Fig. 10A) and ATF2, a substrate of JNK and p38 kinase, was phosphorylated by JNK2 and p38 kinase (Fig. 10B). The p90RSK Ser381 was phosphorylated by activated ERK2 (Fig. 10C) and JNK2 (Fig. 10, C and D), but not by p38 kinase (Fig. 10D). Furthermore, immunoprecipitated p90RSK was activated in vitro by active ERK1, ERK2, JNK1, and JNK2, but not p38 kinase (Fig. 10E). At the same time, the S6 peptide was not phosphorylated by MAPks (Fig. 10F). These data further suggested that UVA-induced phosphorylation of p90RSK Ser381 occurs through activation of ERKs and JNKs, but not p38 kinase.

B/Akt pathway which lead to cell survival (55) and 2) the Ras-ERK pathway, which is important in cell division and differentiation (56). The p90RSK is known to be a downstream kinase of the Raf-MEK-ERK protein kinase cascade and it contains two kinase catalytic domains, the NTD and CTD (16, 22). The CTD of p90RSK is activated by ERKs, which leads to activation of NTD kinase (16). Thus, p90RSK represents a continuation of the ERKs cascade with two additional protein kinase activities (16). However, purified p90RSK that had been dephosphorylated by treatment with phosphatase in vitro could only be activated partially by incubation with active ERKs (18, 28, 57). This suggests that besides ERKs-dependent pathways, p90RSK may also be activated by ERKs-independent pathways.

Recently, full activation of the NTD kinase of p90RSK was shown to require cooperation of the two kinases: ERKs (58, 59), the CTD kinase of p90RSK (16), and PDK1 (3-phosphoinositide-dependent protein kinase-1, a newly identified downstream kinase of the Raf-ERK pathway which lead to cell survival (55) and 2) the Ras-ERK pathway, which is important in cell division and differentiation (56). The p90RSK is known to be a downstream kinase of the Raf-MEK-ERK protein kinase cascade and it contains two kinase catalytic domains, the NTD and CTD (16, 22). The CTD of p90RSK is activated by ERKs, which leads to activation of NTD kinase (16). Thus, p90RSK represents a continuation of the ERKs cascade with two additional protein kinase activities (16). However, purified p90RSK that had been dephosphorylated by treatment with phosphatase in vitro could only be activated partially by incubation with active ERKs (18, 28, 57). This suggests that besides ERKs-dependent pathways, p90RSK may also be activated by ERKs-independent pathways.

Fig. 6. Basal and phosphorylated 90RSK is markedly attenuated in knockout JnK+/− and JnK−/− cells. Preparation and treatment of primary embryo fibroblasts from the mice with wild-type (JnK+/+) and knockout JnK−/− or JnK+/− genes, and analysis of p90RSK S6 kinase activity and phosphorylation at Ser381 and Thr362/Ser381, as well as JNKs and ERKs and their phosphorylated proteins, were performed as described above.

Fig. 7. DNM-JNK1 blocks UVA-induced Ser381 phosphorylation and activation of p90RSK. JB6 Cl 41 cells and Cl 41 stable transfectants, DNM-JNK1, were treated as described in the legend to Fig. 4. The phosphorylated p90RSK, JNKs, and ERKs proteins, as well as p90RSK S6 kinase activity, were determined as described above.
UVA Induces p90\textsuperscript{RSK} Phosphorylation via ERKs and JNKs

Fig. 8. PD169316 but not SB202190 completely blocks UVA-induced phosphorylation and activation of p90\textsuperscript{RSK}. JB6 CI 41 cells were treated as described in the legend to Fig. 3. The cell samples were harvested 30 min after UVA irradiation. Phosphorylated p90\textsuperscript{RSK} and its S6 kinase activity, as well as phosphorylated JNKs, p38 kinase, and ERKs, were analyzed as described under “Experimental Procedures.” The sample membrane was stripped and reprobed with different antibodies. This is one of three independent similar experiments. The figure shows that PD169316, a novel inhibitor of JNKs and p38 kinase (49, 50), completely blocks UVA-induced phosphorylation of p90\textsuperscript{RSK} Ser\textsuperscript{381} (A), JNKs and p38 kinase, but not ERKs (B). SB202190 at 0.5 \(\mu\)M only inhibited phosphorylation of p38 kinase, but did not inhibit phosphorylation of p90\textsuperscript{RSK} Ser\textsuperscript{381} (A), JNKs or ERKs (B). C, data are presented as the mean and standard deviation of six assay samples from three independent experiments and the results show that UVA-stimulated p90\textsuperscript{RSK} activation was significantly attenuated by pretreatment with PD169316 (\(\ast\ast\), \(p < 0.01\)), but not with SB202190 (\(p > 0.10\)) prior to UVA irradiation compared with corresponding treatment with UVA only.

(28, 29). This notion is further supported by the results of Frödin et al. (31) who reported that Ser\textsuperscript{381} phosphorylation may create a docking site that recruits and activates PDK1 and induce a conformational change of p90\textsuperscript{RSK} that facilitates PDK1-mediated Ser\textsuperscript{221} phosphorylation contributing to p90\textsuperscript{RSK} activation (31). These previous studies suggest that Ser\textsuperscript{381} phosphorylation is an important rate-limiting step of p90\textsuperscript{RSK} activation. In our study, Ser\textsuperscript{381} phosphorylation is shown to be induced by UVA irradiation and correlates with UVA-stimulated p90\textsuperscript{RSK} S6 kinase activity. However, little is known regarding whether ERK-independent pathways are involved in Ser\textsuperscript{381} phosphorylation of p90\textsuperscript{RSK}. Like Ser\textsuperscript{221}, Ser\textsuperscript{381} phosphorylation may also be regulated through the PI 3-kinase pathway, inasmuch as UVA-stimulated p90\textsuperscript{RSK} activity and Ser\textsuperscript{381} phosphorylation are blocked by a PI 3-kinase inhibitor (Fig. 3E and data not shown). Moxham et al. (43) indicated that JNKs may be involved in activation and phosphorylation of RSK3, a p90\textsuperscript{RSK} isoform. We, therefore, examined whether the ERKs and JNKs pathways are involved in UVA-stimulated Ser\textsuperscript{381} phosphorylation and activation of p90\textsuperscript{RSK}.

ERKs are known to be upstream kinases of p90\textsuperscript{RSK} (15, 16, 18, 28, 51, 57). But whether Ser\textsuperscript{381} phosphorylation occurs via activation of ERKs is not clear. Data from our present study showed that ERKs phosphorylation (Fig. 5) and activation (data not shown) were induced by UVA irradiation and involved in UVA-stimulated Ser\textsuperscript{381} phosphorylation and activation of p90\textsuperscript{RSK}.

Fig. 9. p90\textsuperscript{RSK} co-immunoprecipitates with ERKs or JNKs, but not p38 kinase. JB6 Cl 41 cells were cultured for 24 h in 100-mm dishes and then starved for 48 h. Fifteen or 30 min following UVA irradiation (160 \(\text{kJ/m}^2\)), the cells were harvested and lysed in 300 \(\mu\)l of immunoprecipitation buffer A. Nonirradiated cells were used as a negative control. Co-immunoprecipitation and subsequent Western immunoblotting analysis were performed as described under “Experimental Procedures.” The upper three panels (A) show that nonphosphorylated p90\textsuperscript{RSK} proteins co-immunoprecipitate with phosphorylated ERKs and JNKs, but not p38 kinases. The lower three panels show that nonphosphorylated ERKs (B) and JNKs (C), but not p38 kinase (D) proteins co-immunoprecipitate weakly with phosphorylated p90\textsuperscript{RSK}. Alternatively, non-immune IgG controls (IP: serum) were negative. P/C indicates total cell lysates as an internal positive control. IP: immunoprecipitation; WB, Western blotting.

Recently, p38 kinase was identified as an upstream kinase of p90\textsuperscript{RSK}-related kinases including PLPK (65), RSK-B (66), and MSK1 (40). Another p90\textsuperscript{RSK} family member, MAPKAP kinase-2, was also shown to be an in vivo substrate of p38 kinase and to be mediated by the mammalian target of rapamycin pathway (68). However, our data showed that p38 kinase may not be involved in UVA-stimulated p90\textsuperscript{RSK} activity and Ser\textsuperscript{381} phosphorylation. We provide evidence that a selective p38 kinase inhibitor, SB202190, and DNMS-p38 had no effect on p90\textsuperscript{RSK} Ser\textsuperscript{381} phosphorylation and its kinase activity induced by UVA. Although the studies of Lian et al. (46) and Horstmann et al. (67) suggested that the p38 kinase inhibitor-sensitive pathway was involved in activation of S6 kinases, our data suggest that UVA-induced Ser\textsuperscript{381} phosphorylation may not be dependent on p38 kinase. The discrepancy may be related to cell type and the kind of stimuli.

Recently, RSK3 was reported to be activated by JNKs both in...
vivo and in vitro (43). Here, we also found that activation of JNKs may be involved in UVA-stimulated p90RSK phosphorylation and subsequent Western immunoblotting analysis, as well as, in vitro assay for activation of immunoprecipitated p90RSK by MAPks, were performed as described under “Experimental Procedures.” Activity of ERKs, JNKs, and p38 kinase was tested by incubation with their substrates, fusion protein Elk1 (for ERK2) or ATF2 (for JNK2 and p38 kinase). Total lysates from SDS lysis of CI 41 cells were used as internal controls in the in vitro p90RSK phosphorylation assays. Incubation of immunoprecipitated p90RSK with no MAPks was used as a negative control in the in vitro p90RSK activation assays. At the same time, incubations of S6 peptide with MAPks, but without immunoprecipitated p90RSK, were performed as internal control experiments. The figure shows that ERK2 phosphorylates Elk1 (A), JNK and p38 kinase both phosphorylate ATF2 (B), and ERK2 and JNK2 (C), but not p38 kinase (D), phosphorylate immunoprecipitated p90RSK proteins at Ser381. The lower panel (E) shows that the S6 kinase activity found in immunoprecipitates of p90RSK that were incubated with active ERK1, ERK2, JNK1, or JNK2 in vitro was significantly greater (*, p < 0.05; **, p < 0.01) than the S6 kinase activity of p90RSK in the absence of MAPks, but the p90RSK activity induced by incubating with p38 kinase is not different from that by incubating with no p38 kinase (p > 0.10). F, shows that in the absence of p90RSK, incubation of the S6 peptide with each of the MAPks in vitro did not induce phosphorylation that was significantly different from the control level (p > 0.10). Data are presented as the mean and standard deviation of five assay samples from three independent experiments (E and F).

Moreover, ERKs, but not JNKs or p38 kinase, is shown to interact with the COOH-terminal tails of three p90RSK isoenzymes (RSK1, RSK2, and RSK3) (51), indicating that a docking site for ERKs, but not for JNKs, is located in the COOH-terminal tail of p90RSK. However, we observed that JNKs, like ERKs, but not p38 kinase, co-immunoprecipitated with p90RSK following UVA exposure, suggesting that a binding site of JNKs is most likely located with another region of p90RSK. This notion was supported further by the evidence from our in vitro studies showing that Ser381 was phosphorylated by active JNK2 and ERK2, but not p38 kinase. Furthermore, S6 kinase activity in the immunoprecipitated p90RSK was activated by JNKs and ERKs, but not p38 kinase in vitro (Fig. 10E), consistent with the suggestions of Moxham et al. (43) that JNKs are required for p90RSK activation. Taken together, our results strongly support the hypothesis that activation and phospho-
ylation of p90RSK Ser381 by UVA is mediated ERKs and JNKs and not by p38 kinase.

Acknowledgments—We thank Dr. Masaaki Nomura for help on the assays for the p90RSK activity and Andria Hansen for secretarial assistance.

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\textit{J. Biol. Chem.} 2001, 276:14572-14580.
doi: 10.1074/jbc.M004615200 originally published online January 31, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M004615200

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