Phosphorylation of Tyr705 and Ser727 of signal transducer and activator of transcription 3 (STAT3) is known to be required for maximal activation by diverse stimuli. Tyr705 phosphorylation is generally accepted to be mediated by the Janus kinase family. But the mechanism for STAT3 (Ser727) phosphorylation is not well understood. Here, we provide evidence that UVA-induced phosphorylation of STAT3 at Ser727 is inhibited by pretreatment of JB6 cells with PD98059 or SB202190. Phosphorylation of STAT3 (Ser727) is also markedly prevented by a dominant negative mutant of ERK2, c-Jun N-terminal kinase 1 (JNK1), or p38 kinase and in knockouts Jnk1—/— or Jnk2—/— cells. Furthermore, STAT3 (Ser727) phosphorylation is suppressed by C- or N-terminal “kinase-dead” mutants of mitogen- and stress-activated protein kinase 1 (MSK1), a downstream kinase of ERKs and p38 kinase, and H89, a potential MSK1 inhibitor. In vitro experiments showed that active MSK1 and JNKs, but not ERKs or p38 kinase, phosphorylate STAT3 (Ser727). Additionally, the role of MAPKs in mediating UVA-stimulated DNA binding activity of STAT3 was investigated. Overall, these results suggest that UVA-induced Ser727 phosphorylation of STAT3 may occur through MSK1 and JNKs.

Signal transducer and activator of transcription 3 (STAT3) was identified as a latent transcription factor that transduces signals to the nucleus and activates expression of many genes in response to cytokines and growth factors (1–4). STAT3 activation is generally accepted to occur by cytokine-stimulated Tyr705 phosphorylation (3, 5, 6). Tyr705 phosphorylation of STAT3 by Janus kinases, receptors with an intrinsic tyrosine kinase activity (e.g. epidermal growth factor receptor (EGFR)), or nonreceptor tyrosine kinases (e.g. Src or Abl) (5–8) appeared to be all that was required for its activation. However, other earlier studies showed that cytokine-stimulated STAT3 signaling activation required a secondary phosphorylation modification at the serine/threonine residues, possibly by H7-sensitive kinase (9) or the extracellular signal-regulated kinases (ERKs) (10). Later, a serine phosphorylation of STAT3, as well as STAT1, 4 and 5, was found to be induced in a stimulus-related manner (4). Further, phosphorylation of STAT3 at Ser727, like Tyr705, was shown to be essential for its full activation (11–14). Additionally, STAT3 signaling activation by environmental stresses, such as heat and osmotic shock, short wave UV light, free radicals, or hypoxia, was also shown to involve induction of multiple signaling pathways (15–17). These findings, therefore, indicate that STAT3 may be a convergent point for integrating signals from multiple pathways (18). More interestingly, only Ser727 phosphorylation of STAT3 was observed to be stimulated by insulin, anisomycin, tumor necrosis factor-α, or arsenite and, to a weaker extent, by NaCl, okadaic acid, or lipopolysaccharide (16, 19, 20). In contrast, Tyr705 phosphorylation was not detected in cells with these treatments (16, 19, 20). These observations indicate that Ser727 phosphorylation may play an important role in mediating the activation of STAT3 independently of Tyr705 phosphorylation. Moreover, Ser727 lies within a potential mitogen-activated protein kinase (MAPK) consensus motif of the C-terminal domain (11, 12, 21). Ser727 phosphorylation is, thereby, postulated to occur through activation of MAPKs, including ERKs, c-Jun N-terminal kinases (JNKs), and p38 kinase (4, 22). However, identification of the kinases responsible for Ser727 phosphorylation has not been fully resolved (4, 23). Therefore, identifying the STAT3 (Ser727) kinases will provide a clearer understanding of the mechanisms of STAT3 activation.

Activation of STAT3 signaling was shown to be involved in regulation of diverse cell processes, including growth, differentiation, proliferation, transformation, as well as apoptosis (24–26). Furthermore, STAT3 was confirmed to be an oncogene (27), and its mediated signaling pathways were initiated by carcinogens (28–30). Solar UV irradiation is believed to be one of the most important skin carcinogens (31, 32). The UV components of sunlight reaching the surface of the earth are UVB (290–320 nm) and UVA (320–400 nm). UVC (200–290 nm) is completely absorbed by the ozone layer of the atmosphere of the earth (31) and, therefore, is unlikely to have major pathophysiological effects. UVB is also partially absorbed by the ozone layer, and efficient protection can be provided by using sunscreen (31, 32). UVA thus constitutes more than 90% of solar UV and is a major contributor to carcinogenesis (31–33). To date, an array of signaling pathways are known to be activated in UV-induced carcinogenesis (34, 35). Recently, STAT1 was reported to be activated through tyrosine phosphorylation in response to UVA (36). However, whether STAT3 signaling in oncogenesis is stimulated by UVA is not clear. In the UVB/UVC response, activation of STAT3 signaling was triggered by phosphorylation at Ser727 but not at Tyr705 (16, 17, 23). Moreover, Ser727 phosphorylation was also shown to be critical for constitutive or aberrant activation of STAT3 signaling in tumorigen...
esi (4, 28, 30, 37). To facilitate an understanding of the role for the STAT3 signaling pathway in UVA-induced skin carcinogenesis, the serine/threonine kinase pathways through which Ser^727 in STAT3 is phosphorylated in UVA-irradiated epidermal JB6 cells were investigated. We provide evidence that UVA-induced Ser^727 phosphorylation of STAT3 may occur through JNKs and mitogen- and stress-activated protein kinase 1 (MSK1), a downstream kinase of both ERKs and p38 kinase.  

MATERIALS AND METHODS  

**Cells and Stable Transfectants**—Mouse epidermal tumor promotion susceptible JB6 Cl 41 cells were cultured in Eagle’s minimum essential medium (Whittaker, Inc., Walkersville, MD) containing 5% fetal bovine serum (FBS), 2 mM t-glutamine, and 25 μg/ml gentamicin at 37°C in humidified air with 5% CO_2_. JB6 Cl 41 cell lines stably transfected with empty CMV-neo vector (CMV-neo) or a dominant negative mutant (DNM) of ERK2, JNK1, or p38 kinase were prepared and identified as described previously (38–43). Transfected JB6 Cl 41 cell lines stably expressing empty CMV5-FLAG vector (CMVS), pCMV-FLAG-wild-type MSK1 (MSK1-WT), pCMV-FLAG-MSK1-A565/C-terminal kinase dead (MSK1-Cd), or pCMV-FLAG-MSK1-A195/N-terminal kinase dead (MSK1-Nd), or pCMV-FLAG-MSK1-A665/C-terminal kinase dead (MSK1-CD) from Dr. D. R. Alessi (Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Scotland, UK) (44) were established and identified according to the recommended protocol of Upstate Biotechnology, Inc. (www.upstate-tech.com) for the stable transfection of CHO cells. The above-mentioned stable transfectants were also grown in 5% FBS with Eagle’s minimal essential medium. Embryonic fibroblasts from wild-type (Egfr^+/+; Jnk^+/+; Jnk2^+/+) and knockout (Egfr^-/-; Jnk1^-/-; or Jnk2^-/-) mice were prepared and identified as previously reported (42, 43, 45). The fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% FBS, 2 mM t-glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin. To reduce a background of protein phosphorylation or activity, the nontransfected and transfected JB6 Cl 41 cells were starved for 24–48 h in 0.1% FBS with minimal essential medium, whereas the embryonic fibroblasts were serum-free Dulbecco’s modified Eagle’s medium prior to treatments.  

**Analysis of Phosphorylated Proteins by Western Blotting**—After starvation, experimental cells were or were not pretreated for 1 h with AG1478, PD135035 (Calbiochem, Inc., San Diego, CA), PD98059, SB202190, rapamycin (Sigma), or H89 ( Alexis Biochemicals, Inc., San Diego, CA) at the doses indicated in the figures and then irradiated with UVA, UVB, or UVC as described previously (42, 43, 46). Here, the nonirradiated cells in the UV box were used as negative controls. The treated cell lysates were harvested in SDS sample buffer and resolved by 8% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes followed by Western blotting analysis with specific antibodies against phospho-STAT3 (Ser^727), phospho-STAT3 (Ty^r^541), phospho-MSK1 (Ser^766), STAT3 (Cell Signaling, Inc., Beverly, MA), or MSK1 (Upstate Biotechnology, Inc., Lake Placid, NY). For a detailed description of the assay see Zhang et al. (42, 43). Additionally, the intensity of the bands in some Western blots was calculated using the Image-Quant Microsoft system or the recommended system (www.totallab.com).  

**In Vitro Assay for STAT3 Phosphorylation by Protein Kinases**—After starvation for 48 h in 0.1% FBS with minimum essential medium, JB6 Cl 41 cells were disrupted in 300 μl of lysis buffer A containing 20 μM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 30 μM Na_2_3_2_, 1 μM leupeptin, and 10 μg/ml aprotinin. The cell lysates were clarified by centrifuging at 17,000  g for 5 min, and then equal amounts of protein were subjected to immunoprecipitation with a STAT3 antibody (42). The immune complex containing STAT3 proteins was incubated at 30°C for 60 min with active ERK1, ERK2, JNK1, JNK2, p38 kinase, or MSK1 (Upstate Biotechnology) in kinase buffer (50 μM Tris-HCl, pH 7.5, 10 mM MgCl_2_, 1 mM EGTA, 1 mM dithiothreitol, 5 mM ATP, and 0.01% Brij 35) (Cell Signaling) after washing twice with lysis buffer A and twice with kinase buffer. Subsequently, Ser^727 phosphorylation of STAT3 was detected by Western blot analysis as described previously (42). Nonphosphorylated STAT3 was used as an internal control to verify equal protein loading. To further evaluate STAT3 phosphorylation catalyzed by MSK1, the immunoprecipitated STAT3 from nonirradiated JB6 cell lysates was incubated with active MSK1 or JNK1 at the indicated doses in the above kinase buffer containing 1 μCi of [γ^32P]ATP. After quantifying by scintillation counting, radioactive phosphate incorporated into immunoprecipitated STAT3 was calculated (www.upstate-tech.com). In addition, to assess the specificity of the phospho-STAT3 (Ser^727) antibody, UVA-irradiated cell lysates were subjected to immunoprecipitation with a phospho-STAT3 (Ser^727) antibody preincubated with a 5-fold concentration of bovine serum albumin (BSA) or STAT3 blocking peptides containing phospho-specific STAT3 (sc-8001P) or Tyr^421 (sc-7993P), a C terminus with no Ser^727 or Tyr^421 sites (sc-482P), or an anti-STAT3 antibody (sc-458). The sample was subjected to Western blotting with the phospho-STAT3 (Ser^727) antibody.  

**MSK1 Activity Assay**—The immune complex MSK1 activity assay with the Akt/SGK peptide as a substrate was employed according to the recommended procedure of Upstate Biotechnology, Inc. (www.upstate-tech.com) (44). In brief, treated or untreated cell lysates were subjected to immunoprecipitation with a STAT3 antibody as reported (42). Then the immune complex containing MSK1 proteins was incubated with agitation for 15 min at 30°C in a mixture of the following: 20 μl of assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na_2_3_2_, and 1 mM dithiothreitol), 10 μl of the immunoprecipitated kinase in kinase buffer, 10 μl of [γ^32P]ATP solution (1 μCi/μl diluted in 75 mM MgCl_2_ and 50 mM α,β-unsaturated ATP). To stop the reaction, the samples were spotted onto a numbered P81 paper square and washed three times (5 min each) with 0.75% phosphoric acid and once (3 min) with acetone. Each sample paper was transferred into a scintillation vial containing 5 ml of scintillation fluid and counted in a β-scintillation counter.  

**STAT3 DNA Binding Mobility Shift Assay**—Electrophoretic mobility shift assays were used to detect STAT3 DNA binding activity after exposure of the cells to UVA irradiation (16, 36). Briefly, 1 h after UVA irradiation, the cells were disrupted in 500 μl of lysis buffer B (50 mM KCl, 0.5% Nonidet P-40, 25 mM Hepes, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 μM dithiothreitol). After centrifugation at 17,000  g for 1 min, the nuclear pellets were washed with the same buffer without Nonidet P-40 and then resuspended in 300 μl of extraction buffer (500 mM KC1 and 10% glycerol with the same concentrations of Hepes, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and dithiothreitol as the buffer B). After an additional centrifugation for 5 min, the supernatant fractions were harvested as nuclear protein extracts and stored at −70°C. The STAT3 oligonucleotide (5'-GATCTCTCGGAGATCCATG-3') probe from Santa Cruz Biotechnology (Santa Cruz, CA) (66) was labeled with [γ^32P]ATP using the Klenow fragment (Life Science Co., Gaithersburg, MD). The nuclear extract (3 μg) was added into the DNA binding buffer containing 5 × 10^5 cpm [γ^32P]-labeled probe, 1.5 μg of poly(dI-dC), and 3 μg of BSA. The reaction mixture was incubated on ice for 10 min followed by an additional incubation at room temperature for 30 min after the DNA-protein complexes were resolved on a 6% non-denaturing polyacrylamide gel. For specific competition, the nuclear extracts were preincubated for 20 min with 2 μg of unlabeled cold probe or for 4 h with 0.2 μg of STAT3 antibody before addition of the labeled STAT3-binding probe. After autoradiography, the radioactivity was quantified using the Image Quant software ( Molecular Dynamics, Sunnyvale, CA).  

**RESULTS**  

**Induction of Ser^727 Phosphorylation of STAT3 by UVA Irradiation**—Ser^727 phosphorylation of STAT3 was shown to be required for its maximal activation by cytokine stimulation (11, 12, 14, 47). A recent report indicated that STAT1, which is highly homologous to STAT3, was activated by UVA irradiation (36), but whether UVA stimulates STAT3 signaling through Ser^727 phosphorylation was yet unidentified. This question was investigated here by using Western blot analysis with a specific antibody to detect phosphorylation of STAT3 at Ser^727. After epidermal JB6 Cl 41 cells were exposed to UVA irradiation, Ser^727 phosphorylation was induced in a dose-dependent (Fig. 1A) and time-dependent (Fig. 1B) manner. Induction of Ser^727 phosphorylation occurred 15 min after irradiation with UVA at 160 J/m^2_ (Fig. 1B), increased to maximal induction at 30 min (Fig. 1), and then gradually decreased to basal level by 120 min, as indicated by the decline in the intensity of the band stained with phospho-STAT3 (Ser^727) antibody.
and a second peak of induction reoccurred at 360 min following irradiation (Fig. 1C). In contrast, a weaker Tyr705 phosphorylation induced by UVA was also observed in the same experiments (Fig. 1, A and B). Tyr705 phosphorylation was induced at 5 min following UVA irradiation and then decreased to basal level at 30 min after irradiation (Fig. 1B). In addition, the phosphorylation at Ser727 (Fig. 1A), but not at Tyr705 (Fig. 1A), was detected in JB6 cells irradiated with UVB or UVC, consistent with previous reports (16, 17, 23, 48). These findings, therefore, suggest that changes in Ser727 phosphorylation of STAT3 may indirectly reflect regulation of STAT3 signaling.

Requirement of EGFR for UVA-induced Ser727 Phosphorylation of STAT3—EGFR-mediated activation of STAT3, but not STAT1, has been shown to be an early event in carcinogenesis (30, 49–51). Furthermore, UVA, like UVB or UVC, was reported to initiate several potential EGFR signaling pathways including phosphatidylinositol 3-kinase and Ras/MAPKs (30, 52, 53). However, although Janus kinase-independent Tyr705 phosphorylation is known to be mediated by EGFR, which has an intrinsic tyrosine kinase activity (7), whether Ser727 phosphorylation of STAT3 by serine/threonine kinases is EGFR-dependent is unclear. To determine this, we used EGFR-deficient JB6 Cl41 cells that were characterized and identified in our laboratory4 and two EGFR-specific tyrosine kinase inhibitors, AG1478 and PD153035 (52, 54, 55). Our data demonstrated that a dose-dependent Ser727 phosphorylation stimulated by UVA irradiation was markedly prevented in Egfr−/− cells compared with wild-type Egfr+/+ control cells (Fig. 2A). Furthermore, UVA-induced Ser727 phosphorylation was inhibited by pretreatment of JB6 cells with either AG1478 or PD153035 (Fig. 2B), but total levels of STAT3 were unchanged in Egfr−/− cells or after pretreatment with EGFR inhibitors (Fig. 2). On the other hand, the phosphorylation of STAT3 at Ser727 was not totally blocked by deficiency of EGFR or inhibition of EGFR kinase (Fig. 2). Therefore, these results indicate that EGFR-dependent and -independent signaling may be involved in Ser727 phosphorylation of STAT3 in response to UVA. In addition, AG1478 and PD153035 had no marked effect on Tyr705 phosphorylation levels (Fig. 1B), based on the observation that the UVA-stimulated increase of Tyr705 phosphorylation did not occur 30 min after irradiation of JB6 cells (Fig. 1, A and B). However, a weaker Tyr705 phosphorylation was induced in UVA-irradiated Egfr−/− cells, but no induction was observed in corresponding control Egfr+/+ cells (Fig. 2A), suggesting that EGFR may be required for UVA-induced Tyr705 phosphorylation.

Involvement of MAPKs in UVA-induced Ser727 Phosphorylation of STAT3—In the UVA response, activation of Raf/ERKS cascades is triggered by EGFR-dependent signaling, but JNKs and p38 kinase activation is not.4 EGFR signaling to STAT3 and subsequent phosphorylation of STAT3 at Ser727 is proposed to be mediated by MAPKs, based on the fact that Ser727 is located in a conserved Pro-Met-Ser-Pro motif of the transcriptional activation domain (11, 12). However, a role of MAPKs in mediating STAT3 is not well elucidated. To assess the role, we first treated JB6 cells with PD98059, an inhibitor of MEK1, which is a kinase that leads to ERKs activation (56), or with SB202190, a specific inhibitor of p38 kinase (57, 58). The results from these experiments showed that UVA-induced Ser727 phosphorylation of STAT3 was markedly suppressed by pretreatment with either PD98059 (Fig. 3A) or SB202190 (Fig. 3B), compared with treatment with UVA only. In addition, induction of STAT3 (Ser727) phosphorylation after exposure of JB6 cells to UVA irradiation was also partially impaired by pretreatment with rapamycin, an inhibitor of mTOR (Fig. 3C).

4 Y. Zhang and Z. Dong, unpublished data.
which was used as an internal control. These observations suggest that besides mTOR (59), Ser727 phosphorylation and maximal activation of STAT3 may be mediated in vivo by ERKs or p38 kinase. However, LY294002 pretreatment had no effect on Ser727 phosphorylation (Fig. 3D), indicating that Ser727 phosphorylation of STAT3 occurred through phosphatidylinositol-3-kinase-independent pathways.

To further determine the effect of MAPKs on Ser727 phosphorylation of STAT3, we prepared and identified JB6 cell lines stably expressing a DNM of ERK2, JNK1, or p38 kinase as previously reported (38–41). UVA-induced phosphorylation of ERKs, JNKs, and p38 kinase was shown to be blocked specifically by DNM-ERK2, DNM-JNK1, and DNM-p38 kinase, respectively (42, 43). Here, the experiments showed that stimulation of Ser727 phosphorylation of STAT3 with UVA was, to different degrees, prevented by expression of DNM-ERK2, DNM-JNK1, or DNM-p38 kinase (Fig. 4B, respectively (42, 43)). These data suggest that besides mTOR (59), Ser727 phosphorylation and maximal activation of STAT3 may be mediated in vivo by ERKs or p38 kinase. However, LY294002 pretreatment had no effect on Ser727 phosphorylation (Fig. 3D), indicating that Ser727 phosphorylation of STAT3 occurred through phosphatidylinositol-3-kinase-independent pathways.

MSK1 Phosphorylation of STAT3 at Ser727 Both in Vivo and in Vitro—To determine an ERK- or p38 kinase-dependent serine/threonine kinase for Ser727 phosphorylation of STAT3, we employed additional in vivo experiments using active MSK1 (44). The results demonstrated that immunoprecipitated STAT3 proteins were phosphorylated at Ser727 by active

![Graph](https://example.com/graph1.png)

**FIG. 3.** Impairment of UVA-induced STAT3 (Ser727) phosphorylation by inhibitors of MEK1, p38 kinase or mTOR kinase. JB6 CI 41 cells were pretreated for 1 h with PD98059 (A), SB202190 (B), rapamycin (C), or LY294002 (D) at the indicated doses prior to UVA irradiation (160 kJ/m²). 30 min after irradiation, the cells were harvested, and then the cell lysates were subjected to Western blot analysis for total and phosphorylated STAT3 (Ser727). The results are representative of at least three independent experiments. p, phosphorylated; np, nonphosphorylated.

![Graph](https://example.com/graph2.png)

**FIG. 4.** Prevention of UVA-stimulated STAT3 (Ser727) phosphorylation by genetic disruption of MAPKs. JB6 CI 41 cell lines stably expressing DNM-ERK2 (A), DNM-p38 kinase (B), DNM-JNK1 (C), or CMV-neo (as an internal control) were harvested at 30 min following irradiation with UVA at the indicated doses. Wild-type (Jnk1+/+) and knockout (Jnk1−/− or Jnk2−/−) cells (D) were treated with UVA (80 kJ/m²) and then harvested at 15 or 30 min after irradiation. Total and phosphorylated STAT3 proteins were determined by Western blotting with specific phospho-STAT3 (Ser727) or Tyr705) or nonphospho-STAT3 antibodies. The data represent one of three independent similar experiments. p, phosphorylated; np, nonphosphorylated.
MSK1 in vitro (Fig. 5B). To further evaluate STAT3 phosphorylation by MSK1 in vitro, we determined catalytic kinetics of MSK1 and JNK1 in kinase assays by using \[^{32}\text{P}\]ATP and immunoprecipitated STAT3 protein. The results in Fig. 5D showed that MSK1 was more effective than JNK1 for catalysis of STAT3 phosphorylation. To examine the role of MSK1 in UVA-induced Ser\(^{727}\) phosphorylation, we prepared JB6 cell lines stably expressing an N-terminal or C-terminal "kinase-dead" mutant (Nd or Cd) of MSK1, as well as wild-type MSK1 (MSK1-WT) (44). As expected, the UVA-stimulated increase in MSK1 activity was significantly inhibited by overexpression of MSK1-Cd or -Nd (\(p < 0.01\), Fig. 6A) compared with MSK1-WT or empty vector CMVS. Furthermore, MSK1-Cd or -Nd markedly prevented UVA-induced Ser\(^{727}\) phosphorylation of STAT3 (Fig. 6B). Additionally, a dose-dependent inhibition of UVA-stimulated Ser\(^{727}\) phosphorylation of STAT3 (Fig. 6C) was observed following pretreatment of JB6 cells with H89, a potential MSK1 inhibitor (60, 61). These data suggest that the Ser\(^{727}\) phosphorylation of STAT3 may be mediated by MSK1 in UVA-irradiated JB6 cells.

MSK1 phosphorylation was shown to reflect its activity indirectly (44, 60). A recent report showed that Ser\(^{376}\) located in the hydrophobic motif of the MSK1 linker region is a critical site for MSK1 activity (62). Here, Ser\(^{376}\) phosphorylation of MSK1 was analyzed by Western blot analysis with a phospho-specific antibody to MSK1 (Ser\(^{376}\)). The data demonstrated...
that UVA-stimulated enhancement of Ser$^{376}$ phosphorylation of MSK1 was completely blocked by pretreatment with PD98059 (A) or SB202190 (B) at the indicated doses. Stable transfectants, DNM-ERK2 (C), DNM-p38 kinase (D), and CMV-neo (as an internal control) were irradiated with 80 or 160 kJ/m$^2$ of UVA. 30 min after irradiation the cells were harvested, and then total and phosphorylated MSK1 proteins were analyzed by Western blotting with nonphospho- or phospho-MSK1 (Ser$^{376}$) antibodies. The results are representative of at least three independent experiments. p, phosphorylated; np, nonphosphorylated.

**FIG. 7.** Inhibition of MSK1 activation and phosphorylation by blocking ERKs or p38 kinase. Before UVA irradiation (160 kJ/m$^2$), JB6 Cl 41 cells were or were not pretreated with PD98059 (A) or SB202190 (B) at the indicated doses. Stable transfectants, DNM-ERK2 (C), DNM-p38 kinase (D), and CMV-neo (as an internal control) were irradiated with 80 or 160 kJ/m$^2$ of UVA. 30 min after irradiation the cells were harvested, and then total and phosphorylated MSK1 proteins were analyzed by Western blotting with nonphospho- or phospho-MSK1 (Ser$^{376}$) antibodies. The results are representative of at least three independent experiments.

**FIG. 8.** Reduction of UVA-stimulated STAT3 DNA binding activity by mutants of MAPKs or MSK1. The experimental cells were harvested 1 h after UVA irradiation. A, the nuclear protein extracts from UVA-irradiated or nonirradiated JB6 Cl 41 CMVS or MSK1-WT cells were or were not preincubated with unlabeled STAT3 probe, STAT3 antibody, or normal serum followed by incubation with labeled probe for STAT3. Furthermore, JB6 Cl 41 cells expressing CMVS, MSK1-WT, MSK1-Cd, or MSK1-Nd (B) or CMV-neo, DNM-ERK2, DNM-JNK1, or DNM-p38 kinase (C) were or were not irradiated with UVA. Then the nuclear proteins were extracted and subjected to electrophoretic mobility shift assay as described under "Materials and Methods." The data are representative of at least three independent experiments.
binding activity of STAT3 (12, 64, 65, 78). To determine the role of MAPK cascades for Ser\textsuperscript{727} phosphorylation in regulating STAT3 DNA binding activity, we analyzed the UVA-induced binding complex in JB6 cell lines expressing MKS1-Cd or -Nd, DNMM-ERK2, DNMM-JNK1, or DNMM-p38 kinase. The results demonstrated that UVA-stimulated enhancement of STAT3 DNA binding activity was significantly abrogated by MKS1 kinase-dead mutants (Fig. 8B). Furthermore, the enhancement was also markedly suppressed in DNMM-ERK2, DNMM-JNK1, or DNMM-p38 kinase cells (Fig. 8C) compared with that observed in control cells. Together with previous reports (12, 64, 65, 78), our data suggest that UVA-induced Ser\textsuperscript{727} phosphorylation of STAT3 may be mediated by ERK- and p38 kinase-dependent MKS1, as well as JNKs, and may be involved in functional regulation of the DNA binding activity of STAT3, but whether serine/threonine sites other than Ser\textsuperscript{727} are involved in this process is not known.

**DISCUSSION**

STAT3 is confirmed to be an oncogene that plays a key signaling role in neoplastic transformation (27). Activation of STAT3 signaling has been increasingly associated with malignant progression (28). Furthermore, STAT3 activation requires phosphorylation of both Tyr\textsuperscript{705} and Ser\textsuperscript{727} in response to stimulation by cytokines and growth factors (4, 11, 63). But, in the UVC or UVB response, only Ser\textsuperscript{727} phosphorylation of STAT3 was detected (16; 23). Induction of a strong Ser\textsuperscript{727} phosphorylation during UVA irradiation was observed here, but Tyr\textsuperscript{705} phosphorylation was weaker. These findings suggest that Ser\textsuperscript{727} phosphorylation may result in STAT3 signaling activation independently of Tyr\textsuperscript{705} phosphorylation in response to some environmental stimuli such as UV stress and thus contribute to UV-induced oncogenesis. This assertion is supported directly from the observation that a dominant negative STAT3 mutant with a change of Ser\textsuperscript{727} to Ala\textsuperscript{727} blocks phosphorylation and inhibits STAT3 signaling activation and Src transformation (27, 28, 67). Therefore, elucidating the signal transduction pathways leading to Ser\textsuperscript{727} phosphorylation of STAT3 will help in understanding the molecular mechanisms involved in activation of STAT3 during UVA-induced carcinogenesis. In addition, this knowledge may lead to the development of novel preventive and therapeutic approaches to intervene in the process. However, the identity of the kinases that are responsible for catalyzing Ser\textsuperscript{727} phosphorylation of STAT3 has been elusive (23, 68). In contrast, Tyr\textsuperscript{705} phosphorylation of STAT3 is a well-characterized event known to be mediated by Janus kinase family kinases and receptor or nonreceptor tyrosine kinases (3, 5–7). In this report, evidence is provided showing that UVA-stimulated Ser\textsuperscript{727} phosphorylation of STAT3 and its DNA binding activity may be mediated by ERK- and p38 kinase-dependent MKS1, as well as JNKs. The UVA-induced Ser\textsuperscript{727} phosphorylation of STAT3 was initiated by EGFR-dependent and -independent signaling pathways. Additionally, a model of the possible signal transduction pathways involved in Ser\textsuperscript{727} phosphorylation of STAT3 is presented in Fig. 9.

Because the site of Ser\textsuperscript{727} phosphorylation, -Pro-Met-Ser-Pro-, present in both STAT1 and STAT3 agrees with the MAPK action consensus sequence, -Pro-Xaa-(Ser/Thr)-Pro- (22), MAPK family members, including ERKs, JNKs, and p38 kinase, are hypothesized to mediate Ser\textsuperscript{727} phosphorylation of STAT3 and regulate its activity in vivo. As expected, in 3T3 cell lysates stimulated with fetal calf serum or epidermal growth factor, a C-terminal peptide of STAT3 was reported to be phosphorylated at Ser\textsuperscript{727} by immune complexes of ERK1 but not p38 kinase or JNK1 (69). Unexpectedly, the homologous C-terminal peptide of STAT1 was a relatively poor substrate for all MAPKs tested both in vitro and in vivo (69). However, our in vitro kinase experiments showed that the Ser\textsuperscript{727} phosphorylation in intact STAT3 immunoprecipitated from quiescent JB6 cell lysates was induced by purified active JNKs but not by ERKs or p38 kinase. This finding agrees with the assumption of Lim and Cao (16) that was drawn from experiments using glutathione S-transferase-STAT3 fusion protein, containing an almost full-length STAT3. The discrepancies of these reactions may be related to their docking interactions contributing to regulation of the specificity and efficiency of the enzymatic reactions with substrates (70, 71). In fact, the specificity of STAT signaling dependent on the SH2 and C-terminal domains was recently confirmed by the experimental evidence of Kovarik et al. (23). Additionally, the occurrences of these reactions may also vary with the molecular conformations in different cell contexts. For example, STAT3 has recently been shown to form stable homodimers independently of Tyr\textsuperscript{705} phosphorylation (72), although STAT3 was thought to exist as monomers in the cytoplasm prior to its activation. Therefore, JNKs may play a direct role in regulation of Ser\textsuperscript{727} phosphorylation of STAT3, whereas the regulation by ERKs and p38 kinase may be mediated indirectly through a serine/threonine kinase (e.g. MSK1).

In fact, studies of STAT1 showed that activation of the p38 kinase pathway was essential for Ser\textsuperscript{727} phosphorylation and subsequent activation of STAT1 and indicated an indirect role for p38 kinase in this process (17, 23, 68, 73). Although most studies on STAT3 Ser\textsuperscript{727} phosphorylation revealed that regulation occurred through activation of ERK-dependent and -independent pathways (65, 69, 74), evidence was not provided as to whether ERKs is a direct Ser\textsuperscript{727} kinase of STAT3. Here, however, we provide evidence that prevention of JNKs activation by DNMM-JNK1 and Jnk1 or Jnk2 deficiency resulted in inhibition of Ser\textsuperscript{727} phosphorylation of STAT3 and its DNA binding activity and that Ser\textsuperscript{727} phosphorylation was induced by active JNKs in vitro, consistent with previous results (16, 75). Thus, JNKs may be a candidate for the direct STAT3 Ser\textsuperscript{727} kinase.

More interestingly, evidence was provided in this report that blocking MSK1 activation by H89, a potential MSK1 inhibitor (60), or a N-terminal or C-terminal kinase-dead mutant of MSK1 (44) resulted in inhibition of UVA-stimulated STAT3 Ser\textsuperscript{727} phosphorylation and its DNA binding activity, indicating that MSK1 may play a role in the process. Furthermore, activation of ERKs and p38 kinase pathways were required for induction of Ser\textsuperscript{727} phosphorylation of STAT3 during stimula-
tion with UV. This was supported by the findings that inhibition of UVA-activated ERKs by PD90859 and DNM-ERK2 and of p38 kinase by SB202190 and DNM-p38 blocked MSK1 phosphorylation and subsequently suppressed STAT3 Ser727 phosphorylation and its DNA binding activity. Moreover, purified active MSK1 can induce phosphorylation at Ser727 in vitro. At the same time, however, the in vitro induction of the Ser727 phosphorylation by active ERK1, ERK2, or p38 kinase was undetectable. Taken together, these findings suggest that the regulation by ERKs and p38 kinase of UVA-induced STAT3 Ser727 phosphorylation and its DNA binding activity may be mediated through MSK1, a direct downstream serine/threonine kinase dependent on both ERKs and p38 kinase. Therefore, MSK1 is likely to be considered as a potential STAT3 Ser727 kinase.

However, the possibility of phosphorylation at other serine/threonine sites cannot be ruled out, because mutation of STAT3 Ser727 with Ala727 blocked phosphorylation and transcriptional activity of STAT3 but did not produce any effect on its DNA binding activity (4, 11). In addition, a relative weaker induction of serine phosphorylation of STAT3 by p38 kinase was shown with radioactive labeled phosphate (75) but was not detected with specific phospho-STAT3 Ser727 antibody in our experiments or in those of Lim and Cao (16). Therefore, the action of MSK1 on STAT3 phosphorylation was postulated to require a prior phosphorylation at other serine/threonine sites that induce a change in its conformation. In fact, a recent study suggests that phosphorylation at other serine/threonine sites may occur by activation of the p38 kinase pathway and play a role in p38 kinase regulation of STAT3 signaling activation (76), because the p38 kinase pathway is required for interferon-α-dependent activation of STATs but not for their Ser727 phosphorylation.

In summary, following integrating signals from multiple signaling pathways (including MAPKs and others) induced concurrently by UV stimulation, STAT3 was phosphorylated at Ser727 and/or other serine/threonine sites, and subsequently STAT3-mediated signaling pathways were activated to regulate expression of target genes. In the UVA response, Ser727 phosphorylation of STAT3 is mediated by JNKs and MSK1, which is dependent upon ERKs and p38 kinase both in vivo and in vitro. The phosphorylation of STAT3 at Ser727 and possibly other serine/threonine sites by MAPKs cascades may play a role in regulating STAT3 DNA binding activity and transcriptional activity as a consequence of integrating multiple signals. In addition, the Ser727 and non-Ser727 phosphorylation events may also require H7-sensitive kinase (9, 65), protein kinase Cδ (77), mTOR kinase (59), or other kinase pathways (37, 78), and thus a goal of our future studies will be to provide a mechanistic explanation for these findings.

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