Ultraviolet B-induced Phosphorylation of Histone H3 at Serine 28 Is Mediated by MSK1

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N-terminal tail phosphorylation of histone H3 plays an important role in gene expression, chromatin remodeling, and chromosome condensation. Phosphorylation of histone H3 at serine 10 was shown to be mediated by RSK2, mitogen- and stress-activated protein kinase-1 (MSK1), and mitogen-activated protein kinases (MAP kinases) in vivo. MAP kinases are involved in ultraviolet B-induced phosphorylation of histone H3 at serine 28 (Zhong, S., Zhong, Z., Jansen, J., Goto, H., Inagaki, M., and Dong, Z., J. Biol. Chem. 276, 12932–12937). However, downstream effectors of MAP kinases remain to be identified. Here, we report that H89, a selective inhibitor of the nucleosomal response, totally inhibits ultraviolet B-induced phosphorylation of histone H3 at serine 28. H89 blocks MSK1 activity but does not inhibit ultraviolet B-induced activation of MAP kinases p70S6k, p90RSK, Akt, and protein kinase A. Furthermore, MSK1 markedly phosphorylated serine 28 of histone H3 and chromatin in vitro. Transfection experiments showed that an N-terminal mutant MSK1 or a C-terminal mutant MSK1 markedly blocked MSK1 activity. Compared with wild-type MSK1, cells transfected with N-terminal or C-terminal mutant MSK1 strongly blocked ultraviolet B-induced phosphorylation of histone H3 at serine 28 in vivo. These data illustrate that MSK1 mediates ultraviolet B-induced phosphorylation of histone H3 at serine 28.

Modification of the N-terminal tail of histone H3 may play a particularly important role in chromatin conformational changes. Increasing evidence indicates that phosphorylation of histone H3 N-terminal serine 10 is closely related to the induction of immediate-early response genes, including proto-oncogenes c-fos and c-jun, and to chromatin remodeling and chromosome condensation during mitosis and meiosis (1–8). Phosphorylation of histone H3 at serine 10 is involved in different signal transduction pathways and is dependent on the specific stimulation or stress. Epidermal growth factor (EGF)1 induces phosphorylation of H3 at serine 10, which is mediated by RSK-2 (2). RSK-2 mutation in humans is linked to Coffin-Lowery syndrome and fibroblasts derived from a Coffin-Lowery syndrome patient fail to exhibit EGF-stimulated phosphorylation of H3 at serine 10 (2). This implies that phosphorylation of histone H3 at serine 10 may be significant in this disease. In addition, mitogen- and stress-activated protein kinase (MSK1) has been shown to mediate EGF or 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced phosphorylation of histone H3 at serine 10 (1). Phosphorylation of histone H3 at serine 10 is consistent with phosphorylation of high mobility group protein 14, which forms the nucleosomal response. The nucleosomal response is closely related to induction of immediate early response genes (1). However, ultraviolet B (UVB) irradiation markedly induces phosphorylation of histone H3 at serine 10 and the phosphorylation was inhibited by PD 98509, a MEK1 inhibitor, SB 202190, a p38 kinase inhibitor, and is blocked in dominant negative mutant-ERK and dominant negative mutant-p38 kinase cells (9). These results showed that MAP kinases mediate UVB-induced phosphorylation of histone H3 at serine 10. Although the understanding of the signal transduction pathways involved in phosphorylation of histone H3 at serine 10 has increased in recent years, the details of the relationship between phosphorylation of histone H3 at serine 10, gene expression, and chromatin remodeling are still unclear and need further study.

Although phosphorylation of histone H3 at serine 10 is related to chromosome condensation, phosphorylation of histone H3 at serine 28 also occurs in chromosomes predominantly during early mitosis and coincides with the initiation of mitotic chromosome condensation in various mammalian cell lines (10). However, the signal transduction pathway mediating histone H3 at serine 28 is unknown. Very recently, we reported that UVB-induced phosphorylation of histone H3 at serine 28 was mediated by ERKs, p38 kinase, and JNK1 (11), but the downstream effectors of these MAP kinases involved in phosphorylation of histone H3 at serine 28 are still unknown. However, the difference in timing between activation of MAP kinases and phosphorylation of histone H3 at serine 28 implies that downstream effectors may mediate UVB-induced phosphorylation of histone H3 at serine 28.

In this article, we used H89, which is a selective inhibitor of the nucleosomal response, to identify the downstream effectors of MAP kinases. H89 totally inhibited phosphorylation of histone H3 at serine 28, but enhanced UVB-induced activation of MAP kinases, p70S6k, p90RSK, and Akt in JB6 cells after...
UVB irradiation. On the other hand, UVB irradiation had no effect on protein kinase A (PKA). Active MSK1 strongly phosphorylated histone H3 at serine 28 in vitro. H89 markedly inhibited MSK1 activity and MSK1 mediated-phosphorylation of histone H3 in vitro. In addition, N-terminal and C-terminal mutants of MSK1 blocked UVB-induced phosphorylation of histone H3 at serine 28 in JB6 cells. Together, these data indicate that MSK1 mediates UVB-induced phosphorylation of histone H3 at serine 28.

EXPERIMENTAL PROCEDURES

Materials—H89 was from Alexis Corp. (San Diego, CA); PD 98059 and SB 202190 were from Calbiochem-Novabiochem Co. (La Jolla, CA); phenylmethylsulfonyl fluoride was from Sigma; pure histone H3 was from Roche Molecular Biochemicals Inc. (Indianapolis, IN); antibody-conjugated alkaline phosphatase (AP) of anti-rabbit IgG and antibodies against phosphorylated ERKs, p38 kinase, and JNKs were from New England Biolabs (Beverly, MA); the antibody against phosphorylated H3 (serine 28) was produced and identified as described previously (10, 11); antibody-conjugated AP of anti-rat IgG was from Pierce Chemical Co. (Rockford, IL); antibodies against β-actin, H3, acetylated H3 (lysine 9), phosphorylated PKA (RII), phosphorylated p90rsk, phosphorylated p70s6k, phosphorylated Akt, active MSK1 and Akt/GSK substrate peptide were from Upstate Biotechnology Inc. (Lake Placid, NY); Eagle’s minimum essential medium (MEM) and fetal bovine serum (FBS) were from QEMINI Bio-products (Calabasas, CA); l-glutamine was from Life Technologies, Inc. (Baltimore, MD); gentamycin was from Quality Biological, Inc.; LipofectAMINE™ 2000 reagent was from Life Technologies, Inc. (Rockville, MD); plasmids of pCMV5-FLAG vector, pCMV5-FLAG-wild-type MSK1, pCMV-FLAG-MSK1 A195-N-terminal kinase-dead, and pCMV5-FLAG-MSK1 A656/C-terminal kinase-dead were kindly provided by Dr. D. Alessi (MRC Oritene Phosphorylation Unit, Dundee, United Kingdom); and polyvinylidene difluoride membrane was from Millipore Corp. (Chicago, IL).

UVB Irradiation—Equivalent numbers of JB6 cells or JB6 cells transfected with the N-terminal mutant MSK1, C-terminal mutant MSK1, or wild-type MSK1 were seeded in 10-cm dishes and cultured in 5% FBS/MEM until they reached 85% confluence. They were then starved in serum-free media and incubated for 45 min at 30 °C for 30 min and then 30 °C with active MSK1 and 200 μM ATP in 50 μl of kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). The samples were resolved by 15% SDS-PAGE and phosphorylated H3 was detected by autoradiography.

Acid-soluble Protein Extraction—After UVB irradiation, cultured cells were harvested and washed twice with cold phosphate-buffered saline. Acid-solution protein extraction was carried out as described by the protocol of Upstate Biotechnology (www.upstatebiotech.com). In brief, acid-soluble proteins were extracted with lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 1.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) and then H2SO4 was added to a final concentration of 0.2 M (0.4 N) and extractions were left for 60 min on ice. Supernatant fractions were transferred to fresh microcentrifuge tubes after centrifugation at 14,000 rpm for 10 min and precipitated on ice for 45 min at 4 °C with a final concentration of 20% trichloroacetic acid. The tubes were centrifuged at 14,000 rpm for 10 min and precipitated on ice for 45 min with a final concentration of 20% trichloroacetic acid. These tubes were centrifuged at 14,000 rpm for 10 min at 4 °C and the pellets were then washed once with acidic acetone and once with acetone. The acid-soluble proteins were stored at −20 °C.

Assay of Phosphorylated H3—Acid-soluble proteins were resolved by 15% SDS-PAGE after boiling for 5 min in SDS sample buffer. Resolved acid-soluble proteins were transferred to polyvinylidene difluoride membranes. Polyvinylidene difluoride membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline for 1 h at room temperature and incubated overnight at 4 °C with the first antibody against either phosphorylated H3 (at serine 28) (10, 11) or total H3. These membranes were then incubated for 4 h at 4 °C with secondary antibodies against rat IgG conjugated-AP or rabbit IgG-conjugated AP, respectively. Membrane bound proteins were detected with enzymatic catalyzed fluorescent substrates (Amer sham Pharmacia Biotech, Piscataway, NJ) and analyzed using the Storm 840 Scanner (Molecular Dynamics Inc., Sunnyvale, CA).

Stable Transfection—Stable transfections were conducted using the LF2000 reagent (from Life Technologies, Inc.) and pCMV5-FLAG vector, pCMV5-FLAG-wild-type MSK1, pCMV5-FLAG-MSK1 A195-N-terminal kinase-dead, or pCMV5-FLAG-MSK1 A656/C-terminal kinase-dead (Dr. D. Alessi, MRC Oritene Phosphorylation Unit). The stable transfection was carried out as described in the protocol from Life Technologies, Inc. (www.lifetech.com/transfer/eltypes/). In brief, 1–3 × 105 JB6 cells were seeded in 6-well plates in 5% FBS/MEM until they reached 85% confluence. For the nontransfected, 10 μg of DNA pCMV5-FLAG vector, pCMV5-FLAG-wild-type MSK1, pCMV5-FLAG-MSK1 A195-N-terminal kinase-dead, or pCMV5-FLAG-MSK1 A656/C-terminal kinase-dead were diluted into 500 μl of serum-free media. LF2000 reagent (20 μl) was diluted into 500 μl of serum-free media and incubated for 5 min at room temperature. Diluted DNA and LF2000 reagent were mixed and incubated for 20 min at room temperature to allow the DNA-LF2000 reagent complexes to form. DNA-LF2000 reagent was then added onto each well and mixed gently by rocking the plates back and forth for 2 min. The cells were incubated at 37 °C for 12 h and then media were changed to fresh growth media. The next day, selective media containing 400 μg/ml G-418 were added until all nontransfected cells were dead resulting in selection of single clone cells. Individual G418-resistant colonies were picked, expanded, and maintained in the presence of G-418 (200 μg/ml). Control cells were transfected with pCMV5-FLAG neo only ("mock"). Individual colonies as well as bulk cultures of G418 selected cells were tested for FLAG-epitope-tagged MSK1 by indirect immunofluorescence staining with a monoclonal FLAG antibody.

MSK1 Activity Assay—Phosphorylation of the Akt/GSK substrate peptide or pure histone H3 by MSK1 was carried out as described by the protocol of Upstate Biotechnology (www.upstatebiotech.com). In brief, the following components were added to a series of tubes: 10 μl of assay dilution buffer (ADB: 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na3VO4, 1 mM dithiothreitol); 10 μl of Akt/GSK substrate peptide or pure histone H3; 10 μl of MSK1 in ADB; and 10 μl of γ-[32P]ATP mixture (1 μCi/μl). Tubes were incubated at 30 °C for 30 min and then 30 °C of the mixture was spotted to individual pieces of p81 paper (2 cm2). The assay squares were washed with 0.75% phosphoric acid and acetone and then transferred to scintillation vials with 5 ml scintillation mixture and counted in a scintillation counter.

Protein Phosphorylation Assay In Vitro—Phosphorylation of pure histone H3 and chromatin by active MSK1 was carried out as described previously (9). In brief, pure histone H3 or chromatin from JB6 cells was incubated for 45 min at 30 °C with active MSK1 and 200 μM ATP in 50 μl of kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). The samples were resolved by 15% SDS-PAGE and phosphorylated H3 was detected by Western blotting with H3-phospho-specific antibodies (10, 11). Nonphosphorylated histone H3 was detected with an antibody against total H3.

RESULTS

UVB Induces Phosphorylation of Histone H3 at Serine 28—To study whether MSK1 mediates UVB-induced phosphorylation of H3 at serine 28, we first exposed mouse epidermal JB6 cells to UVB irradiation and analyzed the phosphorylation of H3 by Western blot using a specific antibody against phosphorylated H3 at serine 28 (10, 11). Acetylation of histone H3 was also detected in the same samples with a specific antibody against acetylation of H3 at lysine 9. A dose-response study showed that phosphorylation of H3 gradually increased with UVB exposures from 1 to 6 kJ/m2 (Fig. 1A). The time course study showed that UVB (4 kJ/m2) induced a rapid and transient phosphorylation of H3 at serine 28 in JB6 C1 41 mouse epidermal cells (Fig. 1B). Phosphorylation of H3 at serine 28 was greater at 30 or 60 min than at 15 or 120 min following UVB irradiation (Fig. 1B) (11). These results indicate that UVB-induced phosphorylation of H3 at serine 28 is dose- and time-dependent. However, acetylation of histone H3 at lysine 9 was unaffected by UVB irradiation (Fig. 1, A3 and B3).

H89 Inhibits UVB-induced Phosphorylation of Histone H3 at Serine 28 in JB6 Cells—H89 (N-(2-(p-bromocinnamylamino)-ethyl)-5-isouquinolinesulfonamide), has an inhibitory action against cyclic AMP-dependent PKA (12). A previous study showed that H89 was a selective inhibitor of the nucleosomal response and inhibited the TPA- or anisomycin-induced phosphorylation of H3 at serine 10 mediated by MSK1 (1). Therefore, H89 provides a useful tool to investigate changes in the
nucleosomal protein, histone H3, following an extracellular stimulation such as UVB irradiation. Our recent study showed that MAP kinases mediated UVB-induced phosphorylation of histone H3 at serine 28 (11). To determine whether H89 affects UVB-induced phosphorylation of histone H3 at serine 28, we pretreated JB6 cells with various concentrations of H89 for 1 h and then exposed the cells to UVB (4 kJ/m²) and incubated for an additional 30 min. The results showed that rather than inhibiting phosphorylation of ERKs, p38 kinase, and JNKs, H89 enhanced UVB-induced phosphorylation of ERKs (Fig. 4, A and C), phosphorylation of p38 kinase was also induced after UVB irradiation, but to a lesser degree (Fig. 3B). The level of phosphorylation of ERKs was higher at 15 than at 30 min after UVB irradiation and was not detected at 60 min. However, phosphorylation of JNKs and p38 kinase was also detected at 30 and 60 min. These results indicate that the time course of activation of MAP kinases (Fig. 3) and phosphorylation of histone H3 at serine 28 induced by UVB irradiation (Fig. 1B) is different.

**H89 Enhances UVB-induced Phosphorylation of ERKs, JNKs, and p38 Kinase, Akt, p70/S6K, or p90RSK in JB6 Cells**—The above experiments indicated that UVB strongly induced phosphorylation of ERKs, p38 kinase, and JNKs. Our recent study indicated that ERK1, ERK2, p38 kinase, and JNK1 are involved in UVB-induced phosphorylation of histone H3 at serine 28 (11). To further determine the role of MAP kinase activation in UVB-induced phosphorylation of histone H3 at serine 28, we used H89 to treat JB6 cells before UVB irradiation and then detected phosphorylation of ERKs, p38 kinase, and JNKs. Our results showed that rather than inhibiting phosphorylation of MAP kinases, 20 μM H89 actually increased UVB-induced phosphorylation of ERKs (Fig. 4A), and to a lesser degree, p38 kinase (Fig. 4B) and JNKs (Fig. 4C). This suggests that H89 may inhibit other protein kinases, which results in the observed repression of UVB-induced phosphorylation of histone H3 at serine 28 (Fig. 2B). Downstream kinases of ERKs include p70/S6K and p90RSK. Akt is an upstream kinase of p70/S6K, JNKs, and p38 kinase is involved in UVB-induced phosphorylation of histone H3 at serine 28 (11). To further determine the role of MAP kinase activation in UVB-induced phosphorylation of histone H3 at serine 28, we used H89 to treat JB6 cells before UVB irradiation and then detected phosphorylation of histone H3 protein (lysine 9), and total histone H3 protein.

**FIG. 1.** UVB strongly induces phosphorylation of H3 at serine 28 in JB6 cells. A, dose-response study: JB6 cells were starved for 48 h by incubation in 0.1% FBS/MEM at 37 °C in a 5% CO₂ atmosphere. Cells were incubated for an additional 2 h in fresh 0.1% FBS/MEM, after which time they were exposed to 1, 2, 4, or 6 kJ/m² of UVB and then incubated an additional 30 min. Phosphorylation of H3 at serine 28 (Phospho-H3-S28) was detected (A1) and analyzed (A2) using the Storm Phospho-Image analysis system (Molecular Dynamics). Acetylation of histone H3 at lysine 9 (Acetyl-H3-L9) was determined by Western blot analysis of acid-soluble nuclear proteins resolved by SDS-PAGE. Total H3 protein was detected in a parallel blot with anti-histone H3 antibodies. The results showed that UVB strongly induced phosphorylation of H3 at serine 28 in a dose-dependent manner (Fig. 2A), and phosphorylated signals of histone H3 at serine 28 (B1, B2), acetylation of histone H3 at lysine 9 (B3), and total H3 protein (B4) were determined as indicated for A. These results reveal that UVB-induced phosphorylation of histone H3 at serine 28 is dose- and time-dependent. The arrows denote the position of phosphorylated histone H3 (serine 28), acetylation of histone H3 (lysine 9), and total histone H3 protein.

**FIG. 2.** H89 inhibits the UVB-induced phosphorylation of H3 at serine 28 in JB6 cells. A, dose-response study: cells were treated with various concentrations of H89 for 1 h and then exposed to UVB (4 kJ/m²). Cells were incubated for an additional 30 min. Total histone H3 protein was detected in a parallel blots with anti-histone H3 antibody. The results showed that UVB induced phosphorylation of histone H3 at serine 28. B, time course study: cells were pretreated with or without 20 μM H89 for 1 h. Then, the cells were exposed to UVB (4 kJ/m²) or not exposed to UVB (control) and incubated an additional 15, 30, 60, or 120 min. H89 (20 μM) blocked phosphorylation of histone H3 at serine 28 at all time points (B1, B2) shows total H3 protein.
Phosphorylation of H3 at Serine 28 Mediated by MSK1

**FIG. 3.** UVB induces phosphorylation of ERKs, p38, and JNKs in JB6 cells. Cells were cultured in 5% FBS/MEM until they reached 85% confluence and then starved in 0.1% FBS/MEM for 48 h. Cells were incubated for an additional 2 h in fresh 0.1% FBS/MEM after which time they were exposed to UVB (4 kJ/m²) and then incubated an additional 15, 30, or 60 min. Media were removed and cells were washed 2 times with cold phosphate-buffered saline. Phosphorylated ERKs, p38 kinase, or JNKs were detected with rabbit anti-phospho-p42/44 MAP kinase, anti-phospho-p38 kinase, or anti-phospho-SAPK/JNK, respectively. Phosphorylation of ERKs (A), phosphorylation of p38 kinase (B), and phosphorylation of JNKs (C) were strongly induced by UVB irradiation in JB6 cells especially at 15 or 30 min. The arrows denote the positions of phosphorylation or total ERKs, p38 kinase, and JNKs proteins.

**FIG. 4.** H89 enhances UVB-induced phosphorylation of ERKs, p38 kinase, JNKs, Akt, p70/s6K, and p90RSK in JB6 cells. JB6 cells were pretreated with 20 μM H89 for 1 h before UVB irradiation and then exposed to UVB (4 kJ/m²), and incubated an additional 15, 30, 60, or 120 min. These results show that 20 μM H89 strongly enhanced UVB-induced phosphorylation of ERKs (A), Akt (D), p70/s6K (E), or p90RSK (F). UVB-induced phosphorylation of p38 kinase (B) and JNKs (C) was also enhanced but to a lesser degree. G, β-actin was used as an internal control to monitor equal protein loading.

but H89 also enhanced UVB-induced phosphorylation of Akt (Fig. 4D), p70/s6K (Fig. 4E), and p90RSK (Fig. 4F) in JB6 cells. These results suggest that UVB-induced phosphorylation of histone H3 at serine 28 does not occur through activation of ERKs, p38 kinase, JNKs, p70/s6K, p90RSK, or Akt and thus implies that other protein kinases are involved.

**UVB Has No Effect on Phosphorylation of PKA in JB6 Cells**—H89 has been shown to have a potent and selective inhibitory action against PKA and also to significantly inhibit cAMP-dependent histone IIb phosphorylation activity (12). To determine whether PKA is involved in UVB-induced phosphorylation of histone H3 at serine 28, we studied the change in phosphorylation of PKA after UVB irradiation. The results showed that in JB6 cells, UVB had no effect on phosphorylation of PKA at any of the time points studied (data not shown). H89, PD 98059, or SB 202190 markedly inhibited phosphorylation of PKA (Fig. 5A) in JB6 cells. On the other hand, both SB 202190 and PD 98059 strongly inhibited UVB-induced phosphorylation of histone H3 at serine 28 in JB6 cells (Fig. 5F). Therefore, H89 target kinases still need to be identified.

**H89 Specifically Inhibits MSK1 Activity in Vitro**—The covalent modification of the N-terminal tail of histone H3 by acetylation, phosphorylation, and methylation regulates transcriptional “on or off” activity (15–17) and influences chromatin remodeling, chromosome condensation, and segregation (18–20). However, these different types of covalent modifications of histone H3 at different sites of the N-terminal tail appear to have various effects in cells. Recently, Rea et al. (18) reported that methylation of lysine 9 interfered with phosphorylation of serine 10 of histone H3, but was also influenced by pre-existing modification in the N terminus of H3. At least one study has shown that MSK1 mediates phosphorylation of histone H3 at serine 10 stimulated by EGF, TPA, or anisomycin (1). Whether MSK1 is involved in UVB-induced phosphorylation of histone H3 at serine 28 is unknown. To investigate the role of MSK1 in phosphorylation of histone H3 at serine 28, we first used Akt/SGK substrate peptide or a pure histone H3 peptide as MSK1 substrates to determine whether H89, PD 98059, or SB 202190 had no effect on PKA phosphorylation of histone H3 at serine 28, we studied the change in phosphorylation of PKA after UVB irradiation. The results showed that in JB6 cells, UVB had no effect on phosphorylation of PKA at any of the time points studied (data not shown). H89, PD 98059, or SB 202190 strongly inhibited UVB-induced phosphorylation of histone H3 at serine 28 in JB6 cells (Fig. 5F). Therefore, H89 target kinases still need to be identified.

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inhibitory effect of H89 on EGF-induced MSK1 activity has indicated that H89 inhibited UVB-induced MSK1 activity. The addition of a pure histone H3 protein with MSK1 in the presence of 200 μM ATP. Phosphorylated histone H3 was detected by Western blotting with a specific antibody for phosphorylated H3 at serine 28, we incubated with active MSK1. A2 and B2 show total H3 protein. The arrows indicate phosphorylated histone H3 at serine 28 and total H3 protein.

SGK substrate peptide, whereas 50 μM PD 98059 and 2 μM SB 202190 did not affect MSK1 activity (data not shown) in vitro. In addition, using a pure histone H3 peptide as substrate, we further determined whether H89, PD 98059, or SB 202190 inhibits MSK1 activity. Results revealed that 20 μM H89, 50 μM PD 98059, or 2 μM SB 202190 all inhibited MSK1 phosphorylation of histone H3 (data not shown) in vitro. These results indicated that H89 inhibited UVB-induced MSK1 activity. The inhibitory effect of H89 on EGF-induced MSK1 activity has been reported by Thomson et al. (1). To confirm that MSK1 specifically phosphorylates histone H3 at serine 28, we incubated pure H3 protein with MSK1 in the presence of 200 μM ATP. The phosphorylation level of H3 at serine 28 was detected with specific antibodies by Western blot analysis (10, 11). Results showed that active MSK1 strongly phosphorylated histone H3 at serine 28 in vitro in an apparent dose-dependent manner (Fig. 6A). Using chromatin isolated from JB6 cells as a substrate for MSK1, we obtained similar results (Fig. 6B), indicating that MSK1 could cause H3 protein phosphorylation at serine 28 of chromatin in vitro.

Inactivation Mutation of MSK1 Blocks UVB-induced Phosphorylation of Histone H3 at Serine 28 in Vivo—The above results indicate that MSK1 may be a mediator of H3 phosphorylation at serine 28. To further confirm that MSK1 has a specific role in UVB-induced phosphorylation of histone H3 at serine 28, we transfected JB6 cells with plasmids containing a pCMV5-FLAG vector, pCMV5-FLAG-wild-type MSK1, pCMV5-FLAG-MSK1 A195-N-terminal kinase-dead, or pCMV5-FLAG-MSK1 A565/C-terminal kinase-dead. MSK1 proteins from these UVB-treated transfected cells were immunoprecipitated using sheep IgG against MSK1 and then a MSK1 kinase assay was performed using [γ-32P]ATP. Immunoprecipitated proteins were from JB6 cells transfected with N-terminal mutant MSK1 (N-MSK1), C-terminal mutant MSK1 (C-MSK1), or wild-type MSK1 (WT-MSK1). Both N-MSK1 and C-MSK1 markedly blocked MSK1-mediated phosphorylation of histone H3 compared with WT-MSK1. The data are presented as mean ± S.E. (n = 2). B, transfected JB6 cells were exposed to UVB and then phosphorylation of histone H3 at serine 28 was analyzed by Western blotting (Fig. 7A) as for Fig. 1 (10, 11). B2 shows total H3 protein. The results revealed that in vivo, both N-MSK1 and C-MSK1 blocked the phosphorylation of histone H3 at serine 28, compared with WT-MSK1. The arrows indicate the phosphorylated histone H3 at serine 28 and total H3 protein.

DISCUSSION

This study further elucidates the signal transduction pathways involved in UVB-induced phosphorylation of histone H3 at serine 28 by focusing particularly on routes by which the phosphorylation of this protein is mediated downstream of MAP kinases. Using H89, a selective inhibitor of the nucleosomial response (1), we found that 20 μM H89 totally inhibited UVB-induced phosphorylation of histone H3 at serine 28. However, H89 actually appeared to enhance UVB-induced activation of ERKs, p38 kinase, JNKs, p70/s6k, p90RSK, and Akt. In contrast, MSK1 activity was markedly inhibited by 20 μM H89 in vitro. Active MSK1 strongly phosphorylated histone H3 at serine 28 and mutant forms of MSK1 blocked UVB-induced phosphorylation of histone H3 at serine 28. These results clearly suggest that MSK1 is a mediator of UVB-induced phosphorylation of H3 at serine 28 in JB6 cells.

N-terminal phosphorylation of histone H3 is highly associated with cell cycle regulation (21–24). Fosfotriecin and okadaic acid initiate premature chromatin condensation and induce H3 phosphorylation (25, 26), but vanadate-induced dephosphorylation of H3 correlates with chromatin decondensation (8). Histone H3 at serine 10 is specifically phosphorylated in mitotic and meiotic chromosome condensation (5, 28). In contrast, the N-terminal tail of histone H3 is not phosphorylated during interphase but becomes phosphorylated at serine 10 just prior to metaphase (25). In mammalian cells, site-specific phosphorylation of H3 at serine 10 occurs during mitosis (6, 7) and various stimuli, including EGF and TPA, and stresses such as

![FIG. 6.](image)

**FIG. 6.** Active MSK1 phosphorylates histone H3 at serine 28 in vitro. Detection of phosphorylation of H3 at serine 28 was carried out at 30 °C for 45 min in the presence of either pure histone H3 or chromatin from JB6 cells and active MSK1, kinase buffer, and 200 μM ATP. Phosphorylated histone H3 was detected by Western blotting with a specific antibody for phosphorylated H3 at serine 28. Pure histone H3 (A) and chromatin (B) were strongly phosphorylated by active MSK1. A2 and B2 show total H3 protein. The arrows indicate phosphorylated histone H3 at serine 28 and total H3 protein.

![FIG. 7.](image)

**FIG. 7.** A dominant negative mutant of MSK1 blocks phosphorylation of histone H3 at serine 28 in vivo. A, following UVB irradiation, MSK1 proteins were immunoprecipitated with sheep IgG against MSK1 and then a MSK1 kinase assay was performed using [γ-32P]ATP. Immunoprecipitated proteins were from JB6 cells transfected with N-terminal mutant MSK1 (N-MSK1), C-terminal mutant MSK1 (C-MSK1), or wild-type MSK1 (WT-MSK1). Both N-MSK1 and C-MSK1 markedly blocked MSK1-mediated phosphorylation of histone H3 compared with WT-MSK1. The data are presented as mean ± S.E. (n = 2). B, transfected JB6 cells were exposed to UVB and then phosphorylation of histone H3 at serine 28 was analyzed by Western blotting (B1) as for Fig. 1 (10, 11). B2 shows total H3 protein. The results revealed that in vivo, both N-MSK1 and C-MSK1 blocked the phosphorylation of histone H3 at serine 28, compared with WT-MSK1. The arrows indicate the phosphorylated histone H3 at serine 28 and total H3 protein.
UV irradiation strongly induce phosphorylation of H3 at serine 10. These phosphorylations were shown to be mediated by RSK2 (2), MSK1 (1), and MAP kinases (9).

A recent study indicated that phosphorylation of histone H3 at serine 28 occurred specifically during early mitosis, at least in mammalian cells and correlated closely with mitotic chromosome condensation (10). However, the biological significance and signal transduction pathway leading to phosphorylation of histone H3 at serine 28 remain unknown. Our recent study showed that MAP kinases are involved in UVB-induced phosphorylation of histone H3 at serine 28 (11). However, UVB-induced H3 serine 28 phosphorylation is time delayed compared with UVB-induced activation of MAP kinases (11), which suggests that downstream effectors of MAP kinases may play an important role in mediating UVB-induced phosphorylation of histone H3 at serine 28. In this study, we used the nucleosome response inhibitor, H89, to explore downstream effectors of MAP kinases after UVB irradiation. H89 could be a very useful probe for investigating the identity of kinases leading to phosphorylation of histone H3 at serine 28. In agreement with the report of Thomson et al. (1), our data indicated that H89 is a MSK1 inhibitor.

UVB-induced phosphorylation of histone H3 at serine 28 was shown to be markedly inhibited by PD 98059 and SB 202190 and dominant negative mutants of ERK2, p38 kinases, and JNK1, indicating that MAP kinases are involved in the phosphorylation of histone H3 at serine 28 (11). Therefore, we determined whether H89 inhibits MAP kinase activation after UVB irradiation. Surprisingly, H89 appeared to enhance UVB-induced activation or phosphorylation of ERKs, p38 kinase, and JNKs (Fig. 4, A-C). These results suggest that H89 may inhibit downstream effectors of MAP kinases. For example, p90RSK is a downstream kinase of ERK1/2 (31–33) and p90RSK has been shown to mediate EGF-induced phosphorylation of histone H3 at serine 10 (2). However, our results indicated that H89 also enhanced p90RSK activation after UVB irradiation in JB6 cells. Other potential candidates for mediating phosphorylation of H3 include the p70 and p85 S6 kinases, which are homologues with a difference of 23 amino acids (34, 35) and are downstream effectors of Akt and ERKs. They contain a bifurcation that produces histone H3-high mobility group-like protein phosphorylation and c-fos/c-jun induction in the nucleus (36). Our results indicated that although UVB induced the activation of Akt and p70/85S6K similar to the results seen with other kinases studied, H89 enhanced this activation (Fig. 4, E and F). These data further suggest that H89 may act on other pathways or downstream effectors of the MAP kinase pathway to repress the UVB-induced phosphorylation of histone H3 at serine 28.

PKA is a prototype serine/threonine kinase that is activated by cAMP (37, 38). PKA phosphorylates an extremely broad range of substrates, including histone H2B and cAMP-response element-binding protein (12, 39, 40), and H89 has been reported to be an inhibitor of PKA (12, 27). Therefore, to determine whether H89 inhibits UVB-induced phosphorylation of histone H3 at serine 28 through the PKA pathway is important. UVB, PD 98059, or SB 202190 had no effect on PKA (Fig. 5A). Furthermore, H89 did not affect PKA phosphorylation in JB6 cells suggesting that other downstream effectors are involved in UVB-induced phosphorylation of histone H3 at serine 28. In agreement with the report of Thomson et al. (1), our data indicated that H89 is a MSK1 inhibitor.

The C-terminal kinase domain of MSK1 is essential for activation of the N-terminal domain and an inactivation mutation either in the N-terminal or C-terminal kinase domain completely abolishes MSK1 activation (30). Endogenous MSK1 is activated by EGF, TPA, or UV irradiation (1, 30). MSK1 is involved in the phosphorylation of several transcription factors (e.g. cAMP-response element-binding protein and ATF1) and nucleosomal components (e.g. histone H3 and high mobility group-14) (1, 30). A recent study indicated that phosphorylation of histone H3 at serine 28 induced by UVB. The arrows indicate activation and ? indicates inhibition. A question mark indicates that the mechanism of the effect is unknown.

**Phosphorylation of H3 at Serine 28 Mediated by MSK1**

![Diagram](http://www.jbc.org/)

**Fig. 8.** MSK1 activation is involved in phosphorylation of histone H3 at serine 28 induced by UVB. UVB induces activation of ERKs, p38 kinase, JNKs, Akt, p70/85S6K, and p90RSK. However, H89 further enhances UVB-induced activation of these kinases but inhibits MSK1 activity resulting in inhibition of UVB-induced phosphorylation of histone H3 at serine 28 induced by UVB. The arrows indicate activation and ? indicates inhibition.
time delay between phosphorylation of histone H3 at serine 28 and activation of MAP kinases may indicate that UVB irradiation first induces activation of MAP kinases and then MAP kinases activate MSK1 to cause phosphorylation of histone H3 at serine 28 forming a signal transduction pathway leading to phosphorylation of histone H3 at serine 28 induced by UVB irradiation (1, 9, 29) (Fig. 8). However, the identity of upstream effectors of MAP kinases involved in UVB-induced phosphorylation of histone H3 at serine 28 still needs to be determined.

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