Mitogen- and stress-activated protein kinases, MSK1 and the closely related isoform MSK2, are nuclear kinases that are activated following mitogen stimulation or cellular stress, including UV radiation, by the ERK1/2 and p38 MAPK signaling cascades, respectively. However, factors that differentially regulate MSK1 and MSK2 have not been well characterized. Here we report that the CK2 protein kinase, which contributes to NF-κB activation following UV radiation in a p38-dependent manner, physically interacts with MSK2 but not MSK1 and that CK2 inhibition specifically impairs UV-induced MSK2 kinase activation. A putative site of CK2 phosphorylation was mapped to MSK2 residue Ser324 and when substituted to alanine (S324A) also compromised MSK2 activity. RNA interference-mediated depletion of MSK2 in human MDA-MB-231 cells, but not MSK1 depletion, resulted in impaired UV-induced phosphorylation of NF-κB p65 at Ser276 in vivo, which was restored by the ectopic expression of MSK2 but not by MSK2-S324A. Furthermore, UV radiation led to the activation of NF-κB-responsive gene expression in MDA-MB-231 cells and induced p65 transactivation capacity that was dependent on MSK2, MSK2 residue Ser324, and p65-Ser276. These results suggest that MSK1 and MSK2 are differentially regulated by CK2 during the UV response and that MSK2 is the major protein kinase responsible for the UV-induced phosphorylation of p65 at Ser276 that positively regulates NF-κB activity in MDA-MB-231 cells.

In response to UV radiation, mammalian cells modulate the activity of several transcription factors, including nuclear factor-κB (NF-κB), which is thought to be an important determinant of cellular survival (1). The most abundant NF-κB complex is a heterodimer composed of p50 and p65 subunits. The p65 subunit harbors the transactivation functions, and p50 can increase inducible DNA-binding ability (2). Under basal conditions, NF-κB is inactive and predominantly cytoplasmic where it is bound to its inhibitor IκB. Following exposure to cellular stress, IκB is phosphorylated, which leads to its degradation. This results in the nuclear translocation and activation of NF-κB p65/50 (2).

There are two distinct signaling pathways leading to the phosphorylation and subsequent degradation of IκB: one that is IκB kinase-dependent and one that is independent of IκB kinase. The IκB kinase-independent pathway is unique in that it is activated following UV-C radiation, and IκB is phosphorylated by the protein kinase CK2 (3). CK2 is a highly conserved and ubiquitous serine/threonine kinase that has been traditionally described as a constitutively active kinase (4). However, several studies have demonstrated that CK2 kinase activity can be stimulated following UV-C radiation in a p38-dependent manner and have shown that CK2 is important in the regulation of cell survival following cellular stress (3, 5–7). CK2 is composed of two catalytic (αα′, α′α) subunits and two regulatory (ββ) subunits that assemble to form the active holoenzyme (8). CK2β dimerization is required for holoenzyme formation (9). CK2 has been shown to phosphorylate, interact with, and regulate other proteins, including other protein kinases (4, 10). Many of these CK2 substrates are thought to be initially bound by the CK2-regulatory subunits, acting as a docking site, which facilitates the subsequent phosphorylation by the catalytic subunits (10).

In addition to the degradation of IκB, the phosphorylation of p65 at Ser276 has been shown to be important for NF-κB transactivation in response to tumor necrosis factor α (TNFα) (11), 12). Furthermore, it has been demonstrated that the mitogen- and stress-activated nuclear protein kinases, MSK1 and the related isoform MSK2, are responsible for the TNFα-mediated stimulation of nuclear p65 Ser276 phosphorylation and transactivation potential (11). As their names suggest, MSK1 and MSK2 are activated following mitogen stimulation or cellular stress, such as UV-C radiation, by the ERK1/2 and p38 kinases, respectively (13, 14). In addition to the phosphorylation of p65 following TNFα stimulation, MSK1 and MSK2 have been shown to specifically phosphorylate CREB, ATF1, and histone H3 and are known to be involved in the activation of the immediate early genes, c-fos and c-jun (15). MSK1 and MSK2 contain
two kinase domains. The C-terminal kinase domain is activated by either p38 or ERK1/2 in response to stimulation. The activated C-terminal kinase then serves to activate the N-terminal kinase domain, which is responsible for substrate phosphorylation. Specifically, the phosphorylation of the MSK2 N-terminal kinase domain at Ser196 by the activated MSK2 C-terminal kinase is essential for MSK2 activation (16). It is not clear whether MSK1 and MSK2 kinase activities undergo differential cellular regulation.

Because MSK1 and MSK2 interact with and are activated by p38 following UV-C radiation, as is CK2, we sought to examine whether CK2 was involved in the regulation of UV-induced MSK1/2 activity. Interestingly, we show that MSK2, but not MSK1, physically interacts with CK2 and undergoes CK2-dependent UV-induced kinase activation. We have identified a putative site of CK2 phosphorylation at serine 324, which is required for maximal activation of MSK2 following UV-C radiation.

Furthermore, we demonstrate that MSK2 is the major kinase responsible for p65-Ser276 phosphorylation and is required for p65 transactivation during the UV response. These results strongly suggest that MSK2 is positively regulated by CK2 and is important for the stimulation of NF-κB activity following UV-C radiation in MDA-MB-231 cells. Significantly, the data also demonstrate for the first time that MSK1 and MSK2 may be activated by distinct signaling pathways.

**EXPERIMENTAL PROCEDURES**

Cloning and Plasmid Constructions—Human MSK1 cDNA (gift from Dr. Peter Cheung) was PCR-amplified and cloned into the pcDNA3.1/V5-His mammalian expression vector by directional TOPO-cloning (Invitrogen). To obtain the full-length human MSK2 cDNA, the following nucleotide sequences were PCR-amplified from human cDNA expressed sequence tags (Open Biosystems). PCR-amplified nucleotides 1–1670 (IMAGE clone ID: 5216639) and nucleotides 1671–2218 (IMAGE clone ID: 2405246) were each cloned into TOPO-TA vectors (Invitrogen). PCR-amplified nucleotides 2219–2316 (IMAGE clone ID: 5763859) were cloned into the pcDNA3.1/V5-His mammalian expression vector by directional TOPO-cloning (2219–2316-pcDNA3.1/V5-His). Enzymatic restriction fragments containing the MSK2 sequences 1–1670 and 1671–2218 were then ligated and subcloned into 2219–2316-pcDNA3.1/V5-His to generate the full-length MSK2 containing mammalian expression vector (MSK2-pcDNA3.1/V5-His) and into pGEX-4T3, yielding pGEX-CK2 and is important for the stimulation of NF-

Cell Culture and Transfections—All cell lines were grown at 37 °C in a humidified atmosphere containing 5% CO2. HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium and MDA-MB-231 cells in RPMI 1640 medium, both supplemented with 10% fetal bovine serum and 50 units of penicillin and 50 μg of streptomycin antibiotics per ml. Transient transfection of plasmids into 293T cells was performed using the Effectene transfection kit (Qiagen) according to the manufacturer’s instructions. Transient transfection of plasmids into MDA-MB-231 cells was performed using the Lipofectamine™ LTX Plus transfection reagent (Invitrogen) according to the manufacturer’s instructions. All siRNA (non-targeting (NT) siRNA pool (D-001206-13), human CK2β siRNA pool (L-007679-00), human MSK1 siRNA pool (M-004665-02), and human MSK2 siRNA (J-004664-06) were purchased from Dharmacon) and esiRNA (non-targeting esiRNA and esiRNA against the 3′-untranslated region of MSK2 was generated according to standard protocols (17)) transfections were performed using Dharmafect1 reagent (Dharmacon) as per the manufacturer’s instructions.

Antibodies—Commercial antibodies used in this study were purchased from R&D Systems (anti-MSK1 goat polyclonal and anti-pS196-MSK2 rabbit polyclonal), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (anti-CK2α goat polyclonal, anti-CK2β rabbit polyclonal and mouse monoclonal, anti-α-tubulin mouse monoclonal, and anti-p65 mouse monoclonal), Abcam (anti-MSK2 rabbit polyclonal and anti-CK2α and anti-CK2α′ rabbit polyclonal), Oncogene (anti-α-tubulin mouse monoclonal), Millipore (anti-HA mouse monoclonal), Cell Signaling Technologies (anti-phospho-p65-Ser276 rabbit polyclonal), and Invitrogen (anti-V5 mouse monoclonal). Anti-V5-agarose affinity gel was purchased from Sigma.

Expression and Purification of Fusion Proteins—GST-CK2β and GST-MSK2 recombinant proteins were produced in Escherichia coli BL21(DE3)/pLysS (Novagen). The expression, extraction, and purification of GST fusion proteins were performed as previously described (18).

Preparation of Cell Extracts, Immunoprecipitation, Pull-downs, and Immunoblotting—For whole cell extract (WCE) preparation, cells were rinsed once in ice-cold phosphate-buffered saline and lysed in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1× Complete mini protease inhibitor mixture (Roche Applied Science), 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, and 50 mM sodium fluoride. For inhibition studies, cells were pretreated with DMSO, DMAT (10 μM), SB203580 (10 μM), or H89 (10 μM) (Sigma) for 2 h at 37 °C. For UV-C treatment, cultured cells were washed once with phosphate-buffered saline and then UV-irradiated (40 or 200 J/m², as indicated) in the presence of phosphate-buffered saline. Following UV irradiation, cells were placed again in culture medium and incubated at 37 °C for specified periods until cell harvest. Pre-treatment with 10 μM anisomycin was performed for 20 min at 37 °C. λ-Protein phosphatase treatments were performed as...
previously described (19). For immunoprecipitations, clarified WCEs were incubated with 1 μg of the relevant antibody on ice for 60 min with occasional gentle agitation, followed by the addition of 30 μl of protein A-coupled Sepharose beads (ThermoFisher) and incubated for an additional 1 h at 4 °C, or WCEs were incubated with V5-agarose (Sigma) at 4 °C for 2 h. Immunoprecipitates were washed three times with lysis buffer, resuspended in SDS-PAGE sample buffer, and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Millipore) and immunoblotted as indicated. Detection was performed using SuperSignal enhanced chemiluminescence (Pierce). For pull-down assays, 1.5 mg of WCE was mixed at 4 °C for 2 h with glutathione-Sepharose beads bound with 1 μg of GST fusion protein. The complexes were then washed and analyzed for associated proteins by immunoblotting as described above.

In Vitro Protein Kinase Assays—MDA-MB-231 or HEK293T cells or those expressing ectopically expressing MSK2-V5 or MSK1-V5 were mock- or UV-irradiated with 200 J/m² UV-C. Thirty minutes post-treatment, cells were harvested as described above, WCEs were incubated with antibodies as indicated, and immunoprecipitates were washed three times with buffer containing 400 mM NaCl and then once with 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 10 mM β-mercaptoethanol. Immunoprecipitation kinase assays were performed with 10 mM MOPS, pH 7.2, 12.5 mM β-glycerol phosphate, 2.5 mM EGTA, 0.5 mM sodium orthovanadate, 0.5 mM dithiothreitol, 6.25 mM magnesium acetate, 62.5 μM cold ATP, 10 μCi of γ-[32P]ATP, and 20 μM crosstide (GRPRTSSFAEG-KK) (Millipore) harboring two lysine residues (underlined) at the C terminus to facilitate binding to P81 paper. The reaction mixtures were incubated for exactly 10 min at 30 °C with constant shaking and then terminated by centrifugation and spotting of the supernatants onto P81 ion exchange paper (Millipore). P81 papers were washed three times for 5 min each with 0.75% phosphoric acid then once with acetone for 5 min. Incorporation of radio-label was measured by scintillation counting. For in vitro CK2 kinase assays, 1 μg of purified recombinant GST or GST-MSK2 was incubated with 10 ng of CK2 (Upstate) in a reaction buffer containing 5 μCi of γ-[32P]ATP for 20 min at 30 °C according to the manufacturer’s instructions. The reaction was stopped by the addition of Laemmli sample buffer, and phosphorylated proteins were visualized by resolving the samples on SDS-PAGE followed by autoradiography.

Reporter Assays—Where indicated, MDA-MB-231 cells were transfected with scrambled esiRNA or MSK2 esiRNA, and 24 h later, cells were transfected with a Gal4-responsive firefly luciferase expression plasmid, pGL2–5xGal4 (gift from Dr. Peter Cheung), the pRL-TK Renilla luciferase normalizing control (Promega), and, as indicated, with pGal4, pGal4-p65, pGal4-p65-S276A, MSK2-V5, MSK2-S232A-V5, or “kinase-dead” MSK2-S196A-V5. 48 h later, cells were mock- or UV-C-irradiated (200 J/m²) in phosphate-buffered saline, medium was replaced, and cells were incubated for 8 h at 37 °C. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Assay System (Promega) as per the manufacturer’s instructions. In all experiments, transfections were performed in triplicate, and the luciferase values were normalized to Renilla luciferase activity.

Quantitative Real-time PCR—Total RNA was extracted from MDA-MB-231 cells using the RNasy minikit (Qiagen). 5 μg of total RNA was reverse transcribed to make cDNA using qScript cDNA SuperMix (Quanta Biosciences). Quantitative real-time PCR was performed with PerfeCTa™ SYBR® Green SuperMix (Quanta Biosciences) as per the manufacturer’s instructions in a 7900HT fast real-time PCR system (Applied Biosystems). Reactions were performed in triplicate and normalized to TATA-box binding protein expression.

Cell Viability Assays—MDA-MB-231 cells expressing NT or MSK2 siRNAs were mock- or UV-C-irradiated (200 J/m²), and cell viability was assessed 24 h later using the colorimetric CellTiter 96® AQueous assay (Promega) by measuring absorbance at 490 nm as per the manufacturer’s instructions.

RESULTS

CK2 Physically Interacts with MSK2 in Human Cells—Both CK2 and MSK1/2 interact with and are dependent on p38 for their activation following UV-C radiation. Therefore, it is possible that CK2 also interacts with and regulates MSK1/2 activity during the UV response. To explore this question, we initially examined whether MSK1 or MSK2 associate with CK2β, because many CK2 substrates may be initially bound by the CK2β regulatory subunit (10). Purified recombinant GST-CK2β or GST immobilized on glutathione-Sepharose beads were mixed with whole cell extracts from human MDA-MB-231 cells in pull-down assays and immunoblotted for endogenous MSK1 and MSK2 (Fig. 1A). As demonstrated in Fig. 1A, GST–CK2β was found to specifically interact with endogenous MSK2, but no detectable interaction was observed with the closely related isoform MSK1. We also noted that UV radiation was associated with decreased MSK2-GST–CK2β interactions, and GST–CK2β appeared to preferentially interact with a faster migrating MSK2 species (Fig. 1A). Co-immunoprecipitation analyses also identified a basal association between MSK2-V5 and ectopically expressed HA2–CK2β immunoprecipitated from HEK293T whole cell extracts but not between HA2–CK2β and MSK1-V5 (Fig. 1B). The reverse co-immunoprecipitation also confirmed an association between endogenous MSK2 but not MSK1 and endogenous CK2β from MDA-MB-231 cell extracts (Fig. 1C). Furthermore, MSK2 co-immunoprecipitated with endogenous CK2α from MDA-MB-231 cell extracts under basal conditions, suggesting that MSK2 interacts with the CK2 holoenzyme. Interestingly, following UV-C radiation, we observed a diminished association between MSK2 and CK2, but the interaction with p38 was maintained (Fig. 1C). There was no detectable interaction between endogenous MSK1 and p38.

MSK2 Activity Is Regulated by CK2 following UV-C Radiation—Given that CK2 appeared to interact with MSK2, we next examined whether CK2 might regulate MSK2 activity following UV-C radiation. To do so, endogenous MSK2 or MSK1 as a negative control was immunoprecipitated from MDA-MB-231 whole cell extracts that had been pretreated with or without the CK2 or p38 inhibitors (DMAT and SB203580, respectively), followed by UV-C radiation, and examined in in vitro kinase
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assays with the peptide substrate, crosstide (Fig. 2A). Although chemical inhibition of p38 reduced both MSK2 and MSK1 UV-induced activation to base-line values as expected, only UV-induced MSK2 activation was affected by CK2 inhibition, with an approximately 3.5-fold reduction (Fig. 2A). When these experiments were repeated in HEK293T cells, similar results were obtained (supplemental Fig. S1). As another means to inhibit CK2 function, we depleted CK2 by siRNA (which impairs CK2 holoenzyme formation and activation (3, 9)) in HEK293T cells and found that this was associated with an approximately 2-fold reduction in MSK2-V5 UV-induced kinase activity but had no effect on MSK1-V5 activity (Fig. 2B). As an additional control, we examined MSK2-V5 activation following anisomycin treatment, which specifically activates the p38 signaling cascade and found that siRNA-mediated CK2 depletion also similarly diminished MSK2-V5 kinase activation (Fig. 2C).

Serine 324 Is Required for UV-induced MSK2 Kinase Activation—The UV-induced MSK2 kinase activity is known to be p38-dependent, and our data suggest that it is also CK2-dependent (Fig. 2). Furthermore, we found that recombinant purified GST-MSK2 was directly phosphorylated by CK2 in vitro (Fig. 3A). We also noted that UV radiation induced an upward MSK2 electrophoretic mobility shift that was abolished by A-protein phosphatase treatment, suggesting that the shift is largely due to MSK2 UV-induced phosphorylation (supplemental Fig. S6). CK2 inhibition partially reduced the UV-induced MSK2 mobility shift, whereas no such effect was noted with MSK1 (Fig. 2A). In contrast, p38 inhibition abolished the UV-induced mobility shifts of both MSK2 and MSK1 (Fig. 2A). CK2 and p38 inhibition had no effect on the migration of MSK2 under basal conditions (Fig. 2A). Collectively, these data suggested that CK2-dependent phosphorylation of MSK2 may modulate UV-induced MSK2 activity.

To determine which amino acid residue(s) of MSK2 might be phosphorylated by CK2, we identified several sites possessing the consensus CK2 phosphorylation motif ((S/T)XXE/D/pS), where pS represents phosphoserine (20). Of these possible sites of CK2 phosphorylation, only Ser\(^{87}\), Ser\(^{119}\), and Ser\(^{324}\) were found to be unique to MSK2 and not conserved in MSK1. Furthermore, of these residues, only Ser\(^{324}\) was found to possess an additional acidic residue at the \(n + 1\) position, which is found in 75% of CK2 sites of phosphorylation (Fig. 3B) (4). Therefore, we initially examined whether Ser\(^{324}\) might be involved in the CK2-dependent regulation of MSK2 kinase activity. To more reliably examine this effect in the context of mammalian cells, we performed site-directed mutagenesis to substitute Ser\(^{324}\) to alanine (S324A) in MSK2-V5 and compared the UV-induced in vitro kinase activity of MSK2-S324A-V5 with that of wild-type (WT) MSK2-V5. In Fig. 3C, in vitro kinase assays of anti-V5 (MSK2) immunoprecipitates from HEK293T cell extracts harvested under basal conditions and following UV-C irradiation demonstrate that the MSK2 mutant protein harboring the S324A substitution was severely impaired for UV-induced kinase activation.

To test whether the phosphorylation of Ser\(^{324}\) might be an important determinant of MSK2 UV-induced kinase activation, Ser\(^{324}\) was also substituted to a phospho-mimicking amino acid (aspartic acid) (S324D) and compared with WT MSK2-V5 in anti-V5 (MSK2) in vitro kinase assays. As shown in Fig. 3C, the phospho-mimicking MSK2 mutant did indeed restore UV-induced MSK2 kinase activity to levels that were comparable with that of WT MSK2-V5.

MSK2 is thought to undergo sequential activation in vivo, whereby p38/ERK first phosphorylates Thr\(^{368}\) within the C-terminal catalytic domain and Ser\(^{341}\) within the linker region (21). Based on mutational analyses, it has been further proposed that the activated C-terminal kinase domain then autophosphorylates Ser\(^{360}\) (within the linker region) and Ser\(^{196}\) (within the activation loop of the N-terminal kinase domain), which is essential for MSK2 activation (21). Therefore, to monitor the effect of Ser\(^{324}\), which lies within the linker region, on MSK2 activation following UV-C radiation, we monitored cell ex-

![FIGURE 1. MSK2 interacts with CK2 in human cells. A, MDA-MB-231 cells treated with or without UV-C radiation (200 J/m\(^2\)) were harvested (30 min later), and WCEs were incubated with recombinant purified GST or GST-CK2β proteins bound to glutathione-Sepharose beads in pull-down assays and immunoblotted for endogenous MSK1 and MSK2 as indicated. * and **, slow and fast migrating MSK2 species, respectively. B, HEK293T cells ectopically expressing HA-CK2β and MSK2-V5, or HA-CK2β and MSK1-V5 were harvested, subjected to anti-HA immunoprecipitation (IP), and then analyzed by immunoblotting with anti-HA (CK2β) and anti-V5 (MSK1 and MSK2) antibodies. C, MDA-MB-231 cells were mock- or UV-C-irradiated, as in A, and WCEs were subjected to anti-MSK1/2 immunoprecipitation and then immunoblotted with MSK1, MSK2, CK2α, CK2β, and p38 antibodies.](image-url)
tracts from HEK293T cells expressing WT, S324A or S324D MSK2-V5 proteins subjected to mock or UV-C radiation and harvested as described in the legend to Fig. 1A. WCEs were immunoprecipitated with anti-MSK2 or anti-MSK1 antibodies, and in vitro MSK2 and MSK1 kinase assays were performed with crosstide and [32P]ATP (left and right histograms, respectively). Activity was measured by scintillation counting. Data were derived from the mean of three independent experiments done in triplicate. Error bars, S.E. Right, Western blotting loading controls for MSK2 and MSK1. B, HEK293T cells were transfected with NT or CK2β-specific siRNAs, and 24 h later, MSK2-V5 or MSK1-V5 constructs were also transfected into the cells. 48 h later, cells were then mock- or UV-C-irradiated and harvested as in A. WCEs were immunoprecipitated with anti-V5 antibody, and in vitro MSK2-V5 and MSK1-V5 kinase assays were performed and measured as in A (top and bottom histograms, respectively). The right panels adjacent to histograms indicate Western blotting loading controls. C, HEK293T cell extracts prepared and analyzed as in C, except that cells were pretreated with or without 10 μM anisomycin instead of UV-C radiation. The bottom panel shows Western blot loading controls.

FIGURE 2. CK2 inhibition impairs UV-induced MSK2 kinase activation in vitro. A, MDA-MB-231 cells were pretreated with DMSO, DMAT (10 μM), or SB203580 (10 μM) for 2 h prior to mock or UV-C radiation and harvested as described in the legend to Fig. 1A. WCEs were immunoprecipitated with anti-MSK2 or anti-MSK1 antibodies, and in vitro MSK2 and MSK1 kinase assays were performed with crosstide and [32P]ATP (left and right histograms, respectively). Activity was measured by scintillation counting. Data were derived from the mean of three independent experiments done in triplicate. Error bars, S.E. Right, Western blotting loading controls for MSK2 and MSK1. B, HEK293T cells were transfected with NT or CK2β-specific siRNAs, and 24 h later, MSK2-V5 or MSK1-V5 constructs were also transfected into the cells. 48 h later, cells were then mock- or UV-C-irradiated and harvested as in A. WCEs were immunoprecipitated with anti-V5 antibody, and in vitro MSK2-V5 and MSK1-V5 kinase assays were performed and measured as in A (top and bottom histograms, respectively). The right panels adjacent to histograms indicate Western blotting loading controls. C, HEK293T cell extracts prepared and analyzed as in C, except that cells were pretreated with or without 10 μM anisomycin instead of UV-C radiation. The bottom panel shows Western blot loading controls.

MSK2-dependent Phosphorylation and Activation of NF-κB

A Phospho-mimicking Substitution at Ser324 Is Resistant to CK2 Inhibition—MSK2 kinase activation is dependent on CK2, and the substitution of Ser324 to a phospho-mimicking amino acid residue relieves MSK2 inhibition following UV-C radiation. Therefore, if the phosphorylation of Ser324 is CK2-dependent, then one would expect that the phospho-mimicking mutant MSK2-S324D would be unaffected by CK2 inhibition. To investigate this possibility further, we compared WT MSK2-V5 and MSK2-S324D-V5 UV-induced activation with or without CK2β-depletion by siRNA in in vitro kinase assays. As shown in Fig. 3E, the ability of WT MSK2-V5 immunoprecipitated from UV-treated cell extracts to phosphorylate crosstide was inhibited by siRNA-mediated CK2β depletion, whereas the kinase activity of MSK2-S324D-V5 was not appreciably affected. These results are consistent with the notion that the phosphorylation of MSK2 at Ser324 is dependent on CK2 in response to UV-C radiation.

MSK2 and MSK1 are closely related isoforms but appear to be differentially regulated by CK2. To further investigate this interesting observation, we noted that Ser324 at the equivalent
position in other mammalian MSK2 proteins was highly conserved, but it was an aspartic acid residue in MSK1 proteins (Fig. 3B). Therefore, we substituted the human MSK1 aspartic acid residue (Asp341), located at the equivalent position to MSK2-Ser324, to alanine and compared this mutant with wild-type MSK1 in in vitro kinase assays. As shown in Fig. 4, substitution of aspartic acid to alanine at amino acid residue 341 had no effect on MSK1 kinase activity, further suggesting that MSK1 and MSK2 are differentially regulated at this position.

**MSK2 Is Required for the UV-induced Phosphorylation of p65 at Ser276 in Vivo**—The mitogen- and stress-activated protein kinases have been shown to phosphorylate the p65 subunit of NF-κB at Ser276 in response to TNFα stimulation and following interleukin-1β treatment (11, 22). Therefore, we wondered whether p65-Ser276 might be phosphorylated by MSK1/2 following UV-C radiation in vivo. To address this question, we first examined whether the UV-induced phosphorylation of p65 at Ser276 in MDA-MB-231 cells (a cell line that has been commonly used in the examination of NF-κB activation (23)) was affected by the inhibition of MSK1/2 (H89), CK2 (DMAT), or p38 (SB203580). Indeed, the immunoblotting of such treated MDA-MB-231 cell extracts with a phospho-specific Ser(P)276 antibody demonstrated that the UV-induced phosphorylation of p65 at Ser276 was undetectable following MSK1/2 or CK2 inhibition.
Because H89 can inhibit both MSK1 and MSK2, we then examined MDA-MB-231 cells depleted of MSK1, MSK2, or both kinases using RNA interference. We found that MSK2 depletion reduced p65-Ser276 phosphorylation following UV-C radiation, whereas the depletion of MSK1 had no detectable effect (Fig. 5B). In addition, when we depleted endogenous MSK2 by esiRNA directed against the 3′-untranslated region and then reconstituted with WT MSK2-V5, MSK2-S324A-V5, or empty vector, we found that only WT MSK2-V5 could rescue the UV-induced phosphorylation of p65 at Ser276 (Fig. 5C). Collectively, our results suggest that Ser276 is important for MSK2 activation and for the in vivo phosphorylation of p65 at Ser276 following UV-C radiation.

**UV-induced p65 Transactivation Is Dependent on MSK2 and p65-Ser276—**To determine the effect of UV-C radiation on NF-κB activity in MDA-MB-231 cells, genes known to be modulated by NF-κB in response to UV radiation (24) were initially examined using quantitative real time PCR (Fig. 6A). We found that UV-C radiation was associated with an increase in the endogenous expression of all such genes examined (Fig. 6A). We then performed luciferase reporter assays whereby p65 was fused to the Gal4 DNA binding element (Gal4-p65), and transactivation was compared with the mutant Gal4-p65-S276A. We found that UV-C radiation was associated with an increase in p65-dependent transactivation capacity, which was virtually abolished when p65-Ser276 was substituted to alanine. To test whether this effect was dependent on MSK2, we depleted MDA-MB-231 cells of MSK2 using RNA interference and similarly did not detect UV-induced p65-dependent transactivation activity (Fig. 6C). Furthermore, expression of WT MSK2-V5 in MDA-MB-231 cells depleted of endogenous MSK2 (by esiRNA directed against the 3′-untranslated region) restored UV-induced p65 transactivation potential, whereas MSK2-S324A-V5 was much less effective (Fig. 6D). The RNA interference-mediated depletion of MSK2 from MDA-MB-231 cells was also found to reduce cellular viability following UV-C radiation (Fig. 6E).

**DISCUSSION**

The related MSK1 and MSK2 protein kinases are activated by mitogens and cellular stress and phosphorylate numerous cellular substrates, including the p65 subunit of NF-κB at Ser276. Indeed, following TNFα stimulation, MSK1/MSK2 double knock-out murine embryonic fibroblasts have been shown to be defective in the phosphorylation of p65 at Ser276 and demonstrate impaired p65 transcriptional activity (11). Many other
studies have also examined MSK1 and MSK2 in the context of double knock-out murine embryonic fibroblasts because of the perceived redundancy of the kinases. Although this may be true in certain instances, our results clearly demonstrate that, following UV-C radiation, MSK2 is the major kinase responsible for p65 phosphorylation at Ser276 and suggest that this is required for subsequent NF-κB activation. Furthermore, we show for the first time that MSK1 and MSK2 can be differentially regulated, specifically by CK2 following UV-C radiation. Our results suggest that maximal UV-induced MSK2 activation relies on the activation of a p38-CK2 signaling pathway in mammalian cells. Several studies have suggested that CK2 lies downstream of p38 (3, 25), and we also found that the UV-induced activation of p38 was unaffected by CK2 inhibition (data not shown). These data are consistent with the notion that a p38-CK2-MSK2 pathway regulates UV-induced MSK2 activity in vivo. We observed that CK2 inhibition with DMAT or siRNA-mediated depletion of CK2 similarly reduced the in vitro UV-induced MSK2 activation on the order of 2–3.5-fold. The substitution of serine 324 to alanine (MSK2-S324A), expected to abolish putative CK2 phosphorylation at serine 324, also resulted in an approximately 3-fold reduction in UV-induced MSK2 activity (Fig. 3).

**FIGURE 6.** MSK2 is required for p65 transactivation and cell survival following UV-C radiation. A, quantitative real-time PCR analysis of endogenous A20, Bcl-xL, and X-IAP gene expression (normalized to TATA-box binding protein) in MDA-MB-231 cells 6 h following mock or UV-C radiation (200 J/m²). B, Gal4, Gal4-p65, or Gal4-p65-S276A transactivation 8 h following UV-C radiation (200 J/m²) in MDA-MB-231 cells co-transfected with pGL2-5 × Gal4 firefly and pRL-TK Renilla luciferase plasmids. Relative firefly luciferase activity was normalized to a Renilla luciferase internal control. C, MDA-MB-231 cells expressing NT or MSK2-specific esiRNAs were transfected with Gal4 or Gal4-p65 and luciferase plasmids. 48 h later, UV-induced transactivation was measured as in B and expressed relative to Gal4 controls. D, MDA-MB-231 cells expressing esiMSK2 were transfected with kinase-dead (KD) MSK2-S196A-V5, MSK2-V5 or MSK2-S324A-V5, Gal4, or Gal4-p65 and luciferase plasmids. 48 h later, cells were mock- or UV-C-irradiated (200 J/m²). Transactivation was measured as in C, and data are expressed relative to MSK2 kinase-dead control. Inset, anti-V5 (MSK2) Western blotting controls from aliquots taken prior to UV treatment. E, MDA-MB-231 cells expressing NT or MSK2-specific siRNAs were mock- or UV-C-irradiated (200 J/m²), and cell viability was determined 24 h later. *, p < 0.001. All error bars represent S.E. derived from three independent experiments done in triplicate.
However, the fact that a phospho-mimicking substitution at Ser\textsuperscript{324} (MSK2-S324D) was resistant to CK2 inhibition suggests that Ser\textsuperscript{324} is the major CK2-dependent site of phosphorylation that modulates MSK2 enzymatic function. In comparison with p38 inhibition, which completely abolished UV-induced MSK2 activity, CK2 inhibition or the MSK2-S324A mutant protein was associated with a smaller reduction in UV-induced MSK2 activity. Therefore, although a CK2-dependent pathway of MSK2 activation probably predominates during the UV response, CK2-independent pathways may also operate.

Serine 324 is evolutionarily conserved in MSK2 homologues (Fig. 3B) and is located within the MSK2 linker region. The linker region harbors several other amino acid residues that have been shown to be essential for MSK2 and MSK1 kinase activation and are sites of either autophosphorylation or p38/ERK phosphorylation (15, 21). Notably, Ser\textsuperscript{324} is not conserved at the equivalent position in MSK1, and the acidic residue at that position (Asp\textsuperscript{341}) does not contribute to MSK1 activation following UV-C radiation (Fig. 4). Our data support a role for the CK2-dependent phosphorylation of Ser\textsuperscript{324}, which is consistent with the direct phosphorylation of recombinant purified MSK2 by CK2 \textit{in vitro} (Fig. 3A). However, it is possible that CK2 may not directly phosphorylate MSK2 \textit{in vivo}, but instead Ser\textsuperscript{324} may be phosphorylated by another CK2-regulated kinase. Alternatively, CK2 may regulate MSK2 autophosphorylation of Ser\textsuperscript{324}. Whether Ser\textsuperscript{324} is important for the regulation of MSK2 kinase activity following other stimuli, such as growth factor stimulation, is not known and is currently under investigation.

We detected constitutive interactions between endogenous MSK2, CK2, and p38 in human cells, and previous studies have also demonstrated basal interactions between MSK2 and p38 and between CK2 and p38 (26, 27). MSK1 has also been reported to interact basally with p38 when ectopically expressed (28), but the strength of this association appears to be weaker than for MSK2-p38 (supplemental Fig. S4). In keeping with these observations, we were able to detect the association of endogenous MSK2 with p38 but not that of MSK1 and p38 (Fig. 1C). Whether this discrepancy is significant \textit{in vivo} or whether the association with CK2 enhances an MSK2-p38 interaction is unclear, but it is a possibility. Collectively, our data are consistent with a model whereby UV-activated p38, contained within a preformed MSK2-CK2-p38 complex, phosphorylates MSK2 and stimulates CK2 to further phosphorylate MSK2 at Ser\textsuperscript{324}, with the subsequent dissociation of CK2 from activated MSK2.

It has been recently shown that the KSR1 scaffold protein binds to the CK2 holoenzyme constitutively and facilitates the CK2-dependent phosphorylation of β-Raf and C-Raf following platelet-derived growth factor stimulation (29). Interestingly, CK2 appears to constitutively phosphorylate B-Raf, suggesting that the basal phosphorylation of B-Raf by CK2 is required for the subsequent activation of B-Raf kinase activity following platelet-derived growth factor stimulation, which in turn leads to the activation of ERK. This CK2-dependent pathway is unlikely to be contributing to our reported observations herein because UV-C radiation does not activate the classical mitogen-activated protein kinase (MAPK) pathway strongly (30), and even if there was a small contribution, one would expect that both MSK1 and MSK2, operating downstream of this pathway, would be affected to similar extents. Indeed, pretreatment with anisomycin, which stimulates the p38 signaling cascade, activated MSK2 in a CK2-dependent manner, which was comparable with what was seen with UV-C radiation (Fig. 2, B and C). Furthermore, chemical inhibition of p38 was found to abolish MSK2 kinase activity to base-line levels (Fig. 2A). Lastly, the weak activation of ERK1/2 following UV-C radiation was unaffected by pretreatment with DMAT in MDA-MB-231 cells (data not shown). Collectively, these data suggest that UV-induced and CK2-dependent MSK2 activation is probably largely attributable to the stimulation of a p38-dependent pathway rather than the Raf-ERK signaling cascade. However, it is possible that the CK2-dependent regulation of Raf kinase activity contributes to the activation of MSK1 and MSK2 following growth factor stimulation via the ERK signaling cascade.

We show that the p65 subunit of NF-κB undergoes UV-induced phosphorylation at Ser\textsuperscript{276} in human MDA-MB-231 cells, and surprisingly, this appears to be primarily dependent on MSK2 with very little contribution from MSK1 (Fig. 5, B and C). In addition to UV-C radiation, MSK1 and MSK2 may be distinctly regulated following other types of stimuli, erasing the notion that these kinases simply perform redundant functions. In keeping with this idea, the TPA- and anisomycin-induced phosphorylation of histone H3 at both serine residues 10 and 28 in primary embryonic fibroblasts has been shown to be largely due to MSK2 and to a lesser extent MSK1 (31).

We found that UV-C radiation resulted in the up-regulation of NF-κB-dependent gene expression and induces NF-κB p65 activation in MDA-MB-231 cells. In contrast, it has been previously suggested that UV-C radiation represses NF-κB-dependent transcription in human U2OS (24). Although this may be a result of cell type-specific differences, it is possible that variations in experimental methods may have contributed. For example, we used full-length wild-type p65 and p65-S276A fused to the Gal4 DNA-binding domain as previously described (11), whereas the data from U2OS cells were obtained using a Gal4-p65 fusion containing only the minimal p65 transactivation domain (residues 428–551) (24). Thus, a contribution from Ser\textsuperscript{276} toward p65 transactivation would not have been detected. Our data suggest that p65-Ser\textsuperscript{276} phosphorylation is required for full p65 activation following UV and that this in turn depends on MSK2 and MSK2-Ser\textsuperscript{324}, because MSK2-S324A-V5 did not rescue p65 transactivation potential to WT levels (Fig. 6D). Indeed, the MSK-dependent phosphorylation of p65-Ser\textsuperscript{276} following TNFα and interleukin-1β treatment has been shown to be required and sufficient for NF-κB activation in murine fibroblasts and human lung fibroblasts, respectively (11, 22). Therefore, the phosphorylation of p65-Ser\textsuperscript{276} following UV-C radiation may be sufficient for the MSK2-dependent activation of NF-κB in MDA-MB-231 cells. However, we cannot exclude the possibility that this depends on the phosphorylation of other p65 sites. The depletion of cellular MSK2 levels by RNA interference was associated with decreased cellular viability, which further suggests that the MSK2-dependent activation of NF-κB following UV radiation is important for the promotion of cell survival in MDA-MB-231 cells \textit{in vivo}. 
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