Induction of a Cell Stress Response Gene RTP801 by DNA Damaging Agent Methyl Methanesulfonate through CCAAT/Enhancer Binding Protein†

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ABSTRACT: RTP801 is a newly discovered stress response gene that is induced by hypoxia and other cell stress signals. Here, we investigated the mechanism by which a DNA damaging agent, methyl methanesulfonate (MMS), induces RTP801 transcription. In HaCaT human keratinocytes, MMS was able to induce a rapid increase in the mRNA level of RTP801. Correspondingly, MMS treatment was capable of stimulating a 2.5 kb RTP801 promoter. Deletion studies with the promoter demonstrated a critical region between −1057 and −981 bp of the promoter that is responsive to MMS treatment. Point mutations of the consensus Elk-1 and C/EBP sites within this region were able to abrogate the stimulatory effect of MMS, indicating that Elk-1 and C/EBP are both involved in the transcriptional regulation of the RTP801 gene by MMS. Furthermore, a gel mobility shift assay revealed that MMS was able to initiate rapid formation of a protein complex that bound the C/EBP site of the promoter. In addition, an anti-C/EBPβ antibody was capable of further shifting the bound protein complex. Therefore, these studies indicate that RTP801 is a transcriptional target of MMS in human keratinocytes and that C/EBP is implicated in transcriptional control of the gene.

RTP801 (REDD1 or dig2) is recently characterized as a novel stress response gene that is responsive to a large variety of extracellular stimuli (*1*−*3*). RTP801 was originally isolated as a hypoxia-inducible factor 1 (HIF-1)[1] responsive gene (*1*). RTP801 is strongly induced by hypoxia both in cultured cells and in an animal model of ischemic stroke (*1*). In addition to hypoxia, RTP801 has been found to be induced by several signals that mediate cell stress, apoptosis, and DNA damage. For example, RTP801 is induced by dexamethasone, thapsigargin, tunicamycin, and heat shock in murine T cell lymphoma cells (*3*). A recent study also indicated that RTP801 is a transcriptional target of p63 and p53 and that it is implicated in reactive oxygen species (ROS) regulation by these two tumor suppressor proteins (*2*). Even though the biological function of RTP801 remains to be defined, increasing evidence from recent studies has suggested that RTP801 is a novel cell stress responsive protein and plays a role in the regulation of cellular ROS production and apoptosis.

However, how RTP801 is regulated at the transcriptional level by cell stress or DNA damage signals remains unclear. In this study, we analyzed the molecular mechanism underlying the transcriptional regulation of the RTP801 gene by a DNA damaging agent, methyl methanesulfonate (MMS). MMS is a strong mutagen and carcinogen that causes DNA damage by alkylation, and such modified DNA is poorly replicated by DNA polymerase. We found that MMS is able to utilize a previously identified cellular pathway to activate the transcription of RTP801.

MATERIALS AND METHODS

Materials, Cell Culture, and Transfection. HaCaT cells, a spontaneously immortalized human epidermal keratinocyte cell line (*4*), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum, 1% nonessential amino acids (GIBCO), 100 μg/mL streptomycin, and 100 units/mL penicillin. MMS was purchased from Sigma. The antibodies against C/EBPα and C/EBPβ were from Santa Cruz Biotechnology.

RNA Isolation and Reverse Transcription PCR (RT-PCR). Total RNA was prepared from HaCaT cells with a RNA isolation kit (Ambion). The RNA was reverse transcribed with oligo(dT) primer using a SuperScript first-strand synthesis system (Invitrogen) to generate the first strand cDNA, followed by PCR to detect the expression of RTP801 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as

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[1] Abbreviations: HIF-1, hypoxia-inducible factor 1; DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MMS, methyl methanesulfonate; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction.

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control. The sequences of the PCR primers are as follows: 5′-TGGGCAAAGAACTACTGCGCCTGG-3′ and 5′-TCAACACTCCTCAATGAGCAGCTG-3′ for human RTP801; 5′-GTCTTCACCACCATGGAGAAGG-3′ and 5′-TCGCTGTTGAAGTCAGAGGAGA-3′ for G3PDH. The PCR products were separated on 2% agarose gel and stained by ethidium bromide (EtBr). The band intensity was determined by the NIH Image program.

Plasmid Construction. The 2.5 kb human RTP801 promoter was cloned by PCR with the human genomic DNA as template using two primers, 5′-CAGCCCTTCCCATTTGAGACAAGTTGTT-3′ and 5′-ATGAACTCAGAGTGCCGGAGCGTAGA-3′. The PCR fragment was first cloned into pCR4Blunt-TOPO (Invitrogen), confirmed by DNA sequencing, and then subcloned into the *Kpn*I and *Sac*I sites of the pGL3-basic luciferase vector (Promega). All deletion constructs of the promoter were generated by the PCR method. The 5′ primers for these constructs are 5′-TGCAGTTCCAGCTTCTCGGGA-3′ (for ΔA), 5′-TGTCAAATGAGAGTAGGGTGCA-3′ (for ΔB), 5′-GTCTGGATCTTTTACACAAAGCG-3′ (for ΔC), 5′-CCCATATCCCGAGCCTTTGCAA-3′ (for ΔD), 5′-CCTTGATCCTCCCCTGCCGCGA (for ΔE), 5′-CCTCTTCCATCAGGGTCCTCCT-3′ (for ΔF), 5′-CCTCTGAGCACTGCTGCCAGGC-3′ (for ΔG), 5′-CCGCCTGAATGATGAAACACGG-3′ (for ΔH), and 5′-CCTGGCTTTTCCAGAGATCTCG-3′ (for ΔI). The 3′ primer used for these deletion constructs was the same as the one for the 2.5 kb promoter. The PCR products were cloned into the pGL3-basic vector for luciferase assay. Point mutations of the RTP801 promoter were introduced into the putative transcription factor binding sites by the PCR method. In short, two PCR fragments were first generated with a point mutation introduced by the PCR primers. These two fragments were annealed and used as a template for PCR with two outside primers. The Elk-1 sequence ATTCCTGTG was replaced by ATTggTGTG, the C/EBP sequence GATGAAACAC by GATGccACAC, and the HNF-4 sequence ATGGCCATTGCA by ATGGatATTGCA. These mutant PCR products were cloned into the pGL3-basic vector and confirmed by DNA sequencing.

Luciferase Assay. HaCaT cells were seeded in 24-well plates at a density of 2×10^5 cells/mL and transfected by SuperFect (Qiagen). A renilla luciferase vector, phRL-SV40 (Promega), was cotransfected as an internal control for transfection efficiency. The cells were harvested at 24−48 h after transfection by lysis with 100 μL of PBS with 0.1% Triton X-100 and 1 mM PMSF. Ten microliters of the lysate was used in the dual-luciferase assay (Promega). The samples were counted for 10 s in a FB12 luminometer (Zylux). A Student's *t*-test was used to analyze the luciferase data by comparing between the untreated samples and the treated ones, respectively.

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed using nuclear extracts from HaCaT cells. The nuclear extracts were prepared as described (*5*). A C/EBP-containing oligonucleotide probe with the sequence of AATGATGAAACACGGGAT based on the RTP801 promoter was used as the probe, which was labeled with [γ-^{32}P]-dATP by T4 polynucleotide kinase. The labeled probes (about 5×10^4 cpm) were incubated with 2 μg of nuclear extract in a buffer containing a final concentration of 4% glycerol, 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 0.1 μg/μL poly(dI-dC). For supershift EMSA, antibodies against C/EBPβ or C/EBPα were added to the binding reaction. The reaction was incubated at 4 °C for 30 min after addition of the probe, separated by electrophoresis with 5% nondenaturing polyacrylamide gel, and detected by autoradiography with the dried gel.

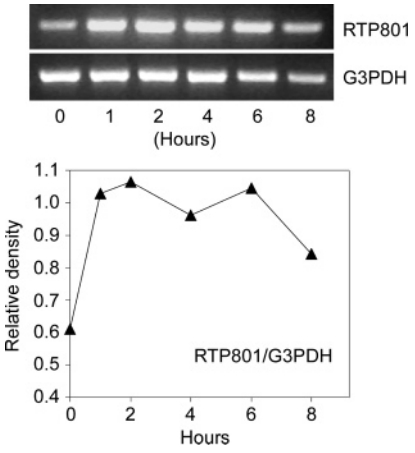

FIGURE 1: Effect of MMS on the mRNA level of RTP801. HaCaT cells were treated with 0.5 mM MMS for various lengths of time. The total RNAs were isolated and used for RT-PCR with specific primers for RTP801 and G3PDH. The PCR products were run in a 2% agarose gel and stained with EtBr (top panel). The relative density of the PCR product of RTP801 was normalized to the G3PDH level and is shown in the lower panel.

RESULTS

MMS Treatment Is Able To Elevate the RTP801 mRNA Level in HaCaT Cells. Recent studies have indicated that RTP801 is a novel stress response gene that is induced by hypoxia and other cell stress signals (*1*−*3*). We first determined whether a DNA damaging agent, MMS, is able to induce RTP801 expression at the mRNA level. This was done in HaCaT cells, a spontaneously immortalized human epidermal keratinocyte cell line (*4*). In the experiment, HaCaT cells were treated with MMS for various lengths of time, and the total RNA was isolated and used in RT-PCR. Primers specific for the human RTP801 cDNA were used in the PCR. The same samples were also used in PCR with primers specific for the housekeeping gene, G3PDH, to monitor the relative RNA amounts used in the experiment. As shown in Figure 1, MMS was not able to cause discernible changes of G3PDH mRNA level. However, MMS was able to induce a rapid upregulation of RTP801 mRNA level. This stimulatory effect reached a maximal level at 1 h after MMS treatment, and such stimulation stayed until 6 h after the treatment. These data provided evidence that MMS is able to regulate RTP801 at the mRNA level in HaCaT keratinocytes.

Regulation of the RTP801 Promoter by MMS. To analyze the transcriptional regulation of RTP801 by MMS, we isolated a 2.5 kb human genomic RTP801 fragment immediately in front of the coding region. This promoter region corresponds to −2548 to +166 bp relative to the transcription initiation site identified by the primer extension assay (data not shown). To determine whether the isolated RTP801 promoter is regulated by MMS, we analyzed the transcriptional activity of the promoter upon MMS treatment. The

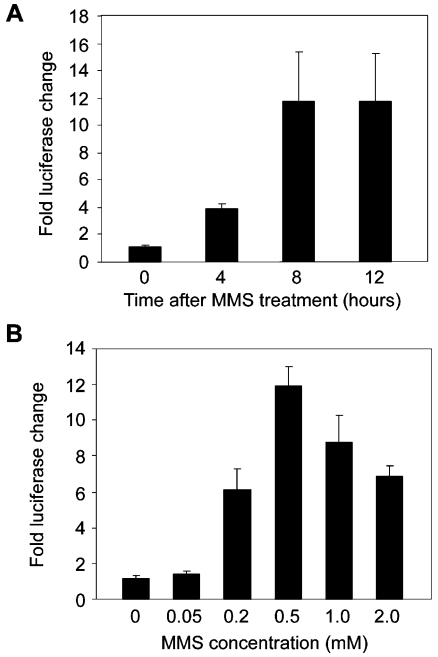

FIGURE 2: Stimulation of the RTP801 promoter by MMS. (A) Effect of various lengths of MMS treatment time on RTP801 transactivation. HaCaT cells were transiently transfected with the RTP801 promoter construct (−2548 bp/+166 bp) with a SV40-driven renilla luciferase plasmid as internal control. Twenty-four hours after transfection, cells were treated with 0.5 mM MMS for different lengths of time. The whole cell lysate was used in the dual-luciferase assay. The fold change of luciferase activity is shown in mean ± SD. (B) Effect of different MMS concentrations. The same promoter construct was used, but the cells were treated with MMS for 8 h in various concentrations as indicated. The fold change of luciferase activity is shown in mean ± SD. At least three independent experiments were performed for the above assays, and only one representative result is shown here.

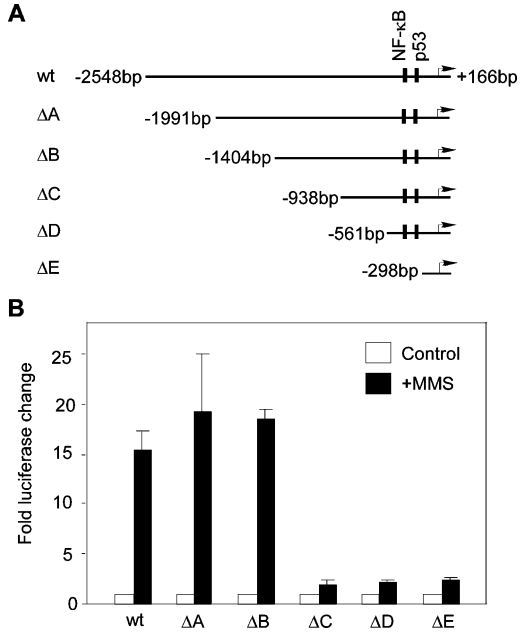

FIGURE 3: Characterization of the human RTP801 promoter. (A) Graphic illustration to show the length of different promoter constructs and the relative location of putative binding sites for NF-κB and p53. (B) Identification of a MMS-responsive region in the RTP801 promoter. HaCaT cells were transiently transfected with various deletion constructs of the RTP801 promoter as indicated and cotransfected with the renilla luciferase vector. Twenty-four hours after transfection, the cells were treated with 5 mM MMS for 8 h, and the fold change of luciferase activity is shown as the mean ± SD.

2.5 kb RTP801 promoter (−2548 to +166 bp) was fused with a firefly luciferase reporter gene. This construct was transiently transfected into HaCaT cells and treated with various concentrations of MMS for different lengths of time. As shown in Figure 2A, MMS was able to stimulate RTP801 promoter activity in a time-dependent manner. We found that MMS treatment for 8 h was able to activate the promoter to the maximal level, equivalent to those treated for 12 h. In addition, we analyzed the effect of different concentrations of MMS on RTP801 transactivation (Figure 2B). At 0.5 mM concentration, MMS was able to activate the promoter to about 12-fold relative to the untreated samples. At concentrations higher than 0.5 mM, the transcriptional stimulation of the promoter appeared to be compromised. This was likely due to elevated cell death at high concentrations of MMS (data not shown). These data, together with our findings that MMS rapidly induced the RTP801 mRNA level by RT-PCR assay (Figure 1), provided convincing evidence that RTP801 is a transcriptional target regulated by MMS in HaCaT keratinocytes.

Identification of a MMS-Responsive Region in the RTP801 Promoter. The human RTP801 promoter contains multiple putative transcription factor binding sites that are potentially involved in the transcriptional regulation of the gene. For example, the promoter contains a putative p53 site around −300 bp (Figure 3A). A recent study also indicated that RTP801 is a transcriptional target of p63 and p53 and such p53 consensus site is likely involved in DNA damage response (*2*). To delineate the region(s) of the RTP801 promoter that is (are) implicated in MMS-mediated activation, we analyzed the effect of MMS treatment on a series of RTP801 promoter deletion mutants which had been fused to the luciferase reporter. As shown in Figure 3, the two deletion constructs, −1991 bp/+166 bp (ΔA) and −1401 bp/+166 bp (ΔB), were responsive to MMS stimulation at a level similar to or even slightly higher than that of the 2.5 kb promoter (−2548 bp/+166 bp). However, three other deletion mutants, −938 bp/+166 bp (ΔC), −561 bp/+166 bp (ΔD), and −298 bp/+166 bp (ΔE), showed a greatly reduced MMS-induced stimulation. These data suggest that the promoter sequences between −1401 and −938 bp likely contain critical regulatory sites that enable the promoter to be responsive to MMS. In addition, the putative NF-κB (around −350 bp) and p53 (around −300 bp) sites do not appear to be directly involved in the MMS-mediated stimulation of RTP801 transactivation in these cells, as deletion of these two sites had little effect on the responsiveness of the promoter to MMS (Figure 3, in comparison between ΔD and ΔE).

To further characterize the MMS-responsive region of the RTP801 promoter, we analyzed a few more deletion mutants of the promoter, including −1158 bp/+166 bp (ΔF), −1057 bp/+166 bp (ΔG), −1019 bp/+166 bp (ΔH), and −981 bp/+166 bp (ΔI), as shown in Figure 4. These constructs were fused with the luciferase reporter and used in promoter assay in HaCaT cells. We found that both the −1158 bp/+166 bp (ΔF) and −1057 bp/+166 bp (ΔG) constructs showed only a slight reduction in MMS-mediated transcriptional stimulation (Figure 4). However, the construct −1019 bp/+166 bp (ΔH) markedly lost the ability to mediate MMS

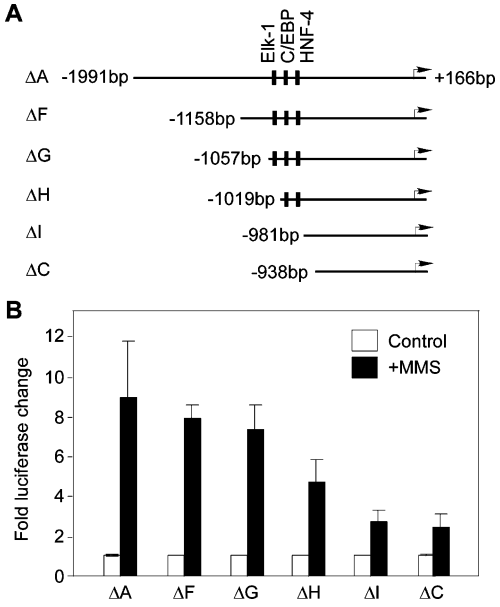

FIGURE 4: Identification of a critical MMS-responsive region. (A) Illustration of promoter constructs used in the experiment. The relative locations of the putative transcription factor binding sites for Elk-1, C/EBP, and HNF-4 are indicated. (B) Effect of different deletions of the RTP801 promoter on the transcriptional effect of MMS. HaCaT cells were transiently transfected with various deletion constructs of the RTP801 promoter as indicated. The cells were treated with 0.5 mM MMS for 8 h. At least three independent experiments were performed. One representative result is shown with the fold change of luciferase activity (mean ± SD).

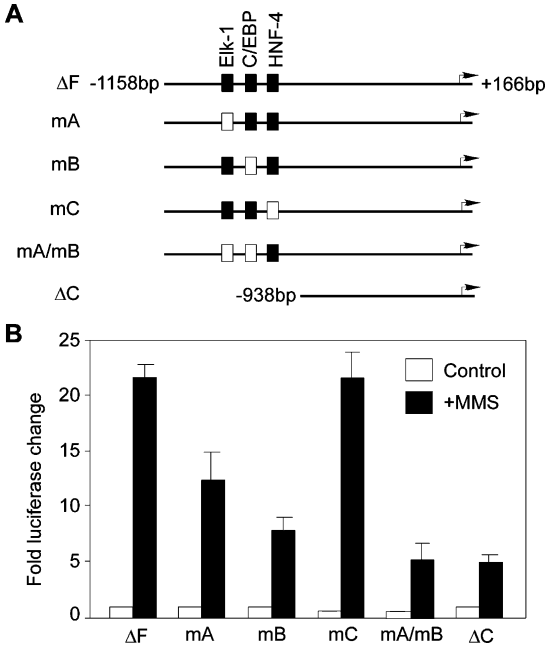

FIGURE 5: Effect of different point mutations of the RTP801 promoter. (A) Graphic illustration to show the relative location of putative transcription factor binding sites for Elk-1, C/EBP, and HNF-4. The filled squares stand for the wild-type sites, and the blank squares are for the mutated ones. (B) Effect of point mutations. HaCaT cells were transiently transfected with various mutation constructs of the RTP801 promoter as indicated, together with the renilla luciferase vector. The cells were treated with 0.5 mM MMS for 8 h at 24 h after the transfection. The fold change of luciferase activity normalized by renilla luciferase activity is shown as the mean ± SD.

activation of the promoter (Figure 4). Furthermore, the −981 bp/166 bp construct (ΔI) only contained a residual ability to mediate MMS stimulation (Figure 4). These data, therefore, further delineated the MMS-responsive region to −1057 to −981 bp of the RTP801 promoter.

Identification of an Elk-1 Site and a C/EBP Site That Mediate MMS Activation of the RTP801 Promoter. As the previous experiments revealed a critical MMS-responsive region between −1057 and −981 bp, we carefully analyzed the putative transcription factor binding sites in this area. There are three major putative transcription factor binding sites that are well conserved in both human and mouse genomic sequences: an Elk-1 site (ATTCCTGTG at −1031 bp/−1023 bp), a C/EBP site (GATGAAACAC at −1009 bp/−999 bp), and a HNF-4 site (ATGGCCATTGCA at −996 bp/−985 bp). Elk-1 is a regulator of c-Fos protooncogene and belongs to the ETS-domain family of transcriptional factors (*6*). It functions as a substrate of MAP kinase and plays an important role in the induction of immediate early gene expression in response to a variety of extracellular signals (*7*). CCAAT/enhancer binding protein (C/EBP) was originally identified as a heat-stable transcription factor in rat liver nuclei and it is able to interact with the CCAAT motif present in several promoters as well as certain viral enhancers (*8*). It belongs to a family of transcription factors that contain a highly conserved basic leucine zipper domain at the C-terminus and plays a pivotal role in the control of cell proliferation, cell differentiation, inflammation, metabolism, and many other cellular responses (*9*). Hepatocyte nuclear factor 4 (HNF-4) is a potent transcriptional activator that controls the expression of a wide variety of genes that are involved in the metabolism of fatty acid, cholesterol, and glucose metabolism (*10*).

To analyze the potential contribution of these sites to the MMS-mediated transcription of RTP801 gene, we introduced point mutations. The Elk-1 sequence ATTCCTGTG was replaced by ATTggTGTG, the C/EBP sequence GATGAAACAC by GATGccACAC, and the HNF-4 sequence ATGGCCATTGCA by ATGGatATTGCA (Figure 5A). In addition, a composite mutation construct was generated, and it contained mutations at both the Elk-1 site and the C/EBP site (Figure 5A). These point mutations were incorporated into the −1158 bp/+166 bp region (ΔF) of the RTP801 promoter and fused with a luciferase reporter before transient transfection in HaCaT cells. As shown in Figure 5B, mutation of the Elk-1 site was able to partially abrogate the MMS-mediated stimulation of the RTP801 promoter. Meanwhile, mutation of the C/EBP site profoundly reduced the MMS-mediated transcriptional activation. Furthermore, mutations of both of these sites were able to almost completely abrogate the responsiveness of the promoter to MMS treatment. On the other hand, mutation of the putative HNF-4 site revealed no obvious effect on MMS-initiated stimulation of the promoter. Taken together, these data provided significant evidence that the Elk-1 and C/EBP sites at the RTP801 promoter are implicated in the transcriptional regulation by MMS.

Characterization of C/EBP Binding by Electrophoresis Mobility Shift Assay (EMSA). We next employed a gel mobility shift assay to further analyze the association of the putative transcription factors to the critical MMS-responsive region that contains the consensus C/EBP site of the RTP801 promoter. As a first step, we analyzed whether MMS treatment is able to induce protein binding to this critical

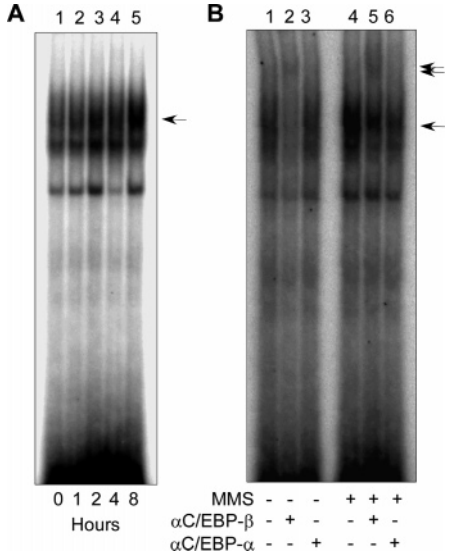

FIGURE 6: Electrophoretic mobility shift assay (EMSA) using nuclear extracts. (A) HaCaT cells were treated with 0.5 mM MMS for various lengths of time as indicated. The nuclear extracts were isolated from the cells and used in EMSA with a ^{32}P-labeled oligonucleotide probe (AATGATGAAACACGGGAT) that contains a C/EBP binding site in the middle. The single arrow indicates the binding complex induced by MMS. (B) Supershift assay with anti-C/EBP antibodies. The nuclear extracts isolated from HaCaT cells treated with 0.5 mM MMS for 8 h were used in EMSA with the oligonucleotide probe containing the C/EBP binding site. The single arrow indicates the band induced by MMS, and the double arrows mark the band shifted by the anti-C/EBPβ antibody (lanes 2 and 5).

region. HaCaT cells were treated with MMS for various lengths of time, and the nuclear extracts were used for EMSA. An oligonucleotide probe (AATGATGAAACACGGGAT) that contains the consensus C/EBP site at the RTP801 promoter was used as a probe in the experiment. As shown in Figure 6A, MMS treatment in these cells was able to induce a time-dependent binding of nuclear proteins to this region, especially at 8 h after the treatment (lane 5). The C/EBP family is composed of at least six members (*9*). Among these members, C/EBPβ has been reported to play an important role in keratinocyte differentiation involving growth arrest and keratin expression (*11*). Therefore, we analyzed if C/EBPβ is present in the protein complex that binds the C/EBP site-containing probe in HaCaT keratinocytes. As shown in Figure 6B, MMS treatment was able to induce a complex binding with the C/EBP site containing probe (lane 4). Interestingly, addition of an anti-C/EBPβ antibody was able to induce a "supershift" in either the absence or presence of MMS (lanes 2 and 5), indicating that C/EBPβ is indeed present in the DNA-binding complex at both basal state and after MMS treatment. We also investigated the possible involvement of another C/EBP member, C/EBPα, and found that a specific antibody for this protein slightly decreased the binding of the nuclear extract to the probe after MMS treatment (lane 6), indicating that C/EBPα might also be present in the complex that bound the consensus C/EBP sequence. In addition, we analyzed EMSA with another oligonucleotide probe (CCAGATTCCTGTGGCCC) that contains the consensus Elk-1 sequence at the RTP801 promoter. However, MMS treatment was not able to induce a protein complex formation with this probe (data not shown). Nevertheless, these data clearly indicated that C/EBP proteins are present in the complex that binds the RTP801 promoter upon MMS treatment.

DISCUSSION

In this study, we investigated the activity of MMS in the transcriptional regulation of RTP801 that is induced by hypoxia and other cell stress signals. In HaCaT human keratinocytes, we found that MMS was able to induce a rapid rise of RTP801 mRNA level. Correspondingly, MMS treatment was capable of stimulating the human RTP801 promoter in HaCaT cells. Through studies with a series of deletion constructs of the promoter, we identified a critical MMS-responsive region between -1057 and -981 bp of the promoter. Further point mutation studies with the putative Elk-1 site and C/EBP site within this region demonstrated that Elk-1 and C/EBP are involved in the transcriptional regulation of the RTP801 gene by MMS. The involvement of C/EBP in the transcriptional regulation by MMS was further confirmed by a gel mobility shift assay. Therefore, these studies provided the first evidence that C/EBP is implicated in the transcriptional regulation of RTP801 upon MMS-mediated DNA damage signals in keratinocytes.

Interestingly, our studies suggest that the putative p53 site in the RTP801 promoter is not directly involved in MMS stimulation of the transcription as the minimal promoter region containing the consensus p53 site was not responsive to MMS treatment (Figure 3, ΔD). In addition, deletion of the p53 site did not affect the responsiveness of the promoter to MMS (Figure 3, ΔE). This is not completely in agreement with the findings that demonstrated RTP801 as a transcriptional target of p63 and p53 (*2*). However, our experiment cannot rule out the possibility that C/EBP may collaborate with p53 to exert transcription control upon DNA damage signals in other types of cells.

One of the interesting findings from this study is that C/EBP is involved in MMS-mediated gene regulation. Our deletion and mutation analyses of the MMS-responsive region indicate that a consensus C/EBP site is implicated in the transcriptional regulation. Furthermore, treatment of HaCaT cells with MMS was able to induce a protein complex that binds the C/EBP consensus sequence, and such binding was "supershifted" by an anti-C/EBPβ antibody. C/EBPs are a family of transcription factors containing a highly conserved basic leucine zipper domain at the C-terminus that participates in dimerization and DNA binding (*9*). At least six C/EBP members have been characterized so far, including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPϵ, and C/EBPζ. These factors play pivotal roles in the control of cell proliferation, cell differentiation, inflammation, metabolism, and many other cellular responses. C/EBP has not been shown to be implicated in DNA damage-mediated gene regulation. Regulation of C/EBP could occur at the transcriptional, translational, and posttranslational levels (*12*). It will be of great importance in the future to uncover at which level C/EBP is involved in MMS-mediated transcriptional control. It is noteworthy that MMS is a general methylating agent that not only methylates DNA but also may have an effect on proteins. Therefore, we cannot rule out the possibility that MMS may directly affect the functions of C/EBP or other proteins to modulate RTP801 transcription. In addition, RTP801 is regulated by other cell stress signals

including hypoxia (*1*). It will be interesting to elucidate if the C/EBP pathway as described here is also involved in the transcriptional regulation of RTP801 by other stimuli.

At present, the biological function of RTP801 is largely unknown. One of the suspected functions of RTP801 is its role in apoptosis. In some types of cells, RTP801 has a protective effect against apoptosis. Overexpression of RTP801 is able to protect MCF7 and PC12 cells from hypoxia- and H_2O_2-triggered apoptosis (*1*). In addition, overexpression of RTP801 in WEHI7.2 murine T lymphoma cells was able to protect the cells from dexamethasone-induced apoptosis (*3*). However, RTP801 may promote cell death in other types of cells. It has been shown that RTP801 is able to increase sensitivity of nondividing PC12 cells to the insults of hypoxia and oxidative stress (*1*). Consistently, liposomal delivery to RTP801 to mouse lungs leads to massive cell death (*1*). In addition, deletion of RTP801 in the mouse is able to attenuate oxygen-induced retinopathy (*13*). In our studies, overexpression of RTP801 is able to induce nuclear condensation as well as apoptosis in HaCaT keratinocytes (data not shown). Therefore, the apoptotic effect of RTP801 seems to be dependent on cellular context. It is likely that RTP801 interacts with the apoptotic machinery differently in a cell-dependent manner. Therefore, detailed biochemical analysis of RTP801 function in the apoptosis pathway in various cell types will greatly aid in clarifying this issue in the future.

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