Critical Role of Cysteine Residue 81 of Macrophage Migration Inhibitory Factor (MIF) in MIF-induced Inhibition of p53 Activity*

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Macrophage migration inhibitory factor (MIF) is a potent modulator of the p53 signaling pathway, but the molecular mechanisms of the effect of MIF on p53 function have so far remained unclear. Here we show that MIF physically interacts with the p53 tumor suppressor in vitro and in vivo. This association was significantly reduced by a C81S mutation but not C57S or C60S mutations, suggesting that Cys81 is essential for the in vivo association between MIF and p53. This association also depended on Cys242 (and, to some extent, on Cys238) within the central DNA binding domain of p53. Ectopic expression of MIF, but not MIF(C81S), inhibited p53-mediated transcriptional activation in a dose-dependent manner. Conversely, knockdown of endogenous MIF stimulated p53-mediated transcription. MIF inhibited p53-induced apoptosis and cell cycle arrest, whereas the MIF(C81S) mutant, which is unable to physically associate with p53, had no effect. Consistent with these findings, confocal microscopy showed that MIF prevented p53 translocation from the cytoplasm to the nucleus. We also demonstrated that MIF suppresses p53 activity by stabilizing the physical association between p53 and Mdm2. These results suggest that MIF physically associates with p53 and negatively regulates p53 function.

The abbreviations used are: MIF, macrophage migration inhibitory factor; siRNA, small interfering RNA; shRNA, short hairpin RNA; DTT, dithiothreitol; Mdm2, mouse double minute 2; FACS, fluorescence-activated cell sorting; GST, glutathione S-transferase; GFP, green fluorescent protein; DBD, DNA binding domain; SFU, S-fluorouracil; HA, hemagglutinin.

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MATERIALS AND METHODS

Reagents and Cell Culture—Anti-GST, anti-FLAG (M2), anti-β-actin, Alexa Fluor-594 anti-mouse, Alexa Fluor-488 anti-rabbit, and anti-histone (H2B) antibodies were described previously (22). The anti-MIF, anti-Mdm2, anti-p53(DO-1), anti-p21, and anti-Bax, anti-histone H2B antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-HA antibody was kindly provided by Dr. S-C. Bae (Chungbuk National University, Cheongju, Korea). 293T, HEK293, MCF7, U2OS, and HCT116 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum as described previously (23).

Generation of Inducible MIF shRNA Cell Lines—For inducible knockdown of endogenous MIF expression, double-stranded oligonucleotides (5'-TCGAGGACACCAACGTGCCC GCCGCTTCAGAGGC CCGGGCAGTGTGTG TTAAA-3', MIF sequence underlined) was cloned into the pSingle-tTS-shRNA vector (Clontech). HCT116 cells were transfected with pSingle-tTS-shRNA harboring MIF-specific shRNA or pSingle-tTS-shRNA empty vector using WelFect-ExTM Plus (WelGENE, Daegu, Korea). Inducible MIF shRNA stable clones were screened in the presence of 450 μg/ml G418 until all control parental HCT116 cells died. Stable clones were isolated and treated with 1 μg/ml doxycycline (Sigma), a tetracycline analogue, for 72 h, and endogenous MIF knockdown was determined by immunoblot analysis using anti-MIF antibody.

Plasmids and DNA Construction—The expression plasmids for wild-type p53 and its deletion derivatives were a kind gift from Dr. S-J Um (Sejong University, Seoul, Korea). To generate the p53(DBD) construct, PCR was performed using the following primers: forward primer (5'-GCGATTTCTTTTGATCTTG GGACA-3') containing an EcoRI site (underlined) and reverse primer (5'-GCCCTCGAGGGCGAGTTCTCTTTCC-3') containing an XhoI site. The amplified PCR products were cut with EcoRI plus XhoI and cloned into a pFLAG-CMV2 vector using EcoRI plus SalI sites to generate the FLAG-p53(DBD). The six Cys to Ser p53(DBD) substitution mutants (C176S, C238S, C176S/C238S, C176S/C242S, C238S/C242S, and C238S/C242S) were amplified using the following mutant primers containing alterations in the nucleotide sequence of p53(DBD): for p53(DBD) Cys176 to Ser (C176S), sense 5'-GGAATTCTTCTTGCATTTGC GCAGTT-3'; for p53(DBD) Cys238 to Ser (C238S), sense 5'-AACTACATGTCAAACAGTTCC-3'; for p53(DBD) Cys238 to Ser (C238S), sense 5'-AACGTTCTCTAAATGCCG GGCC-3'; antisense 5'-GGCAGCATTTGACATGTAGT-3'; for p53(DBD) Cys242 to Ser (C242S), sense 5'-AACGTTCTCTTTCCATGAGG CCCG-3', antisense 5'-GGCCGCCCCATTTGAAGACTT-3'. To generate the double substitution mutant p53(DBD) C176S/C242S, FLAG-C176S was used as the template, and the sense and antisense primers of C242S and p53(DBD) were used for PCR amplification. To generate the double substitution mutants C176S/C242S and C238S/C242S, PCR was carried out using FLAG-C176S and FLAG-C238S as templates in the presence of primers for C242S and p53(DBD). The identity of all of the PCR products was confirmed by nucleotide sequencing analysis on both strands.

In Vivo and in Vitro Binding Assay—Each plasmid DNA indicated under “Results” was transiently transfected into the indicated cells with WelFect-ExTM Plus, according to the manufacturer’s instructions. In vivo binding assays were performed as described previously (23). To determine the ability of p53 to bind to wild-type and mutant forms of MIF in vitro, p53 was translated in vitro using the TnT reticulocyte lysate system as directed by the manufacturer (Promega). In vitro-translated C176S and FLAG-C238S were incubated with recombinant MIF, and anti-MIF antibody was used to identify the ability of p53 to bind to MIF.
FIGURE 2. Effect of cysteine residues of MIF on the MIF-p53 interaction. A, 293T cells were transiently transfected with the appropriate expression plasmids, and GST fusion proteins were purified on glutathione-Sepharose beads (GST purification), resolved by SDS-PAGE, and visualized with ECL. Formation of a complex between MIF and p53 was determined by Western analysis using an anti-FLAG antibody (top panel). The same blot was re-probed with an anti-GST antibody to examine the coprecipitation of equivalent amounts of the GST-fused MIF proteins (middle panel). The expression level of p53 in total cell lysates was analyzed by Western analysis using an anti-FLAG antibody (bottom panel). WB, Western blot. B, MIF-p53 complex formation was determined by native 8% PAGE. In vitro-translated 35S-labeled p53 was prepared with the TNT reticulocyte lysate system. 35S-Labeled p53 was incubated with unlabeled recombinant wild-type MIF (MIF(WT)) and MIF mutants (C57S, C60S, and C81S) in the presence of 5 mM H2O2 at room temperature for 1 h. The procedure was the same as for SDS-PAGE, with the exception that SDS and β-mercaptoethanol were not included in any solutions, and samples were not boiled before loading. C, MIF-depleted extracts (MIF depletion) of HEK293 cells were mixed with recombinant GST alone, GST-MIF(WT), or GST-MIF(C81S) proteins (each 4 μg). The mixtures were then purified on glutathione-Sepharose beads, followed by immunoblot analysis using an anti-p53 antibody to determine the complex formation between MIF and p53 (top panels). The level of immunodepleted MIF in total cell lysates was determined by immunoblot analysis using an anti-MIF antibody (bottom panels). D, HCT116 cells that harbor stably integrated pSingle-tTS-shRNA empty vector (V) or pSingle-tTS-shRNA vector containing MIF-specific shRNA (MIF shRNA) were cultured in the presence or absence of 1 μg/ml doxycycline (Dox) for 72 h to determine the complex formation between recombinant MIF and endogenous p53 proteins (right, top panel). Inducible silencing of endogenous MIF expression by doxycycline was assessed by immunoblotting using an anti-MIF antibody in stable MIF shRNA clones (left, top panel). β-Actin was used as a control. P, parental HCT116 cells; V, vector. E, effect of reductants (DTT and β-mercaptoethanol (β-ME)) and H2O2 on the interaction between endogenous MIF and p53. MCF7 and HCT116 cell lysates treated with the indicated concentration of H2O2, DTT, and β-ME were immunoprecipitated with an anti-p53 antibody (IP: α-p53), followed by immunoblotting with an anti-MIF antibody (top and 4th panels). The amount of immunoprecipitated p53 and the expression level of MIF in total cell lysates were determined by immunoblot analysis using the anti-p53 antibody (2nd and 5th panels) and an anti-MIF antibody (3rd and bottom panels), respectively.
35S-labeled p53 was incubated with unlabeled recombinant wild-type and mutant forms of MIF in the presence of 5 mM H2O2 at room temperature for 1 h, and then assessed by 8% nondenaturing PAGE as described previously (22).

RNA Interference Experiments—RNA interference experiments were performed using MIF-specific siRNAs. The sequences used were as follows: MIF-specific siRNA 1 (5'-ACACCAACGUGCCCGCCGCGd-TdT-3') corresponding to a coding region (amino acids 7–13) of the human MIF (GenBank™ accession number NM002415); MIF-specific siRNA 2 (5'-CCUUCUGGGCCCGCCCGCGd-TdT-3') corresponding to a C-terminal region (nucleotides 427–445); MIF-specific siRNA 3 (5'-GACUCCAACCUUCGCUCGd-TdT-3') corresponding to a 3'-untranslated region (nucleotides 527–545); and a nonspecific control siRNA (5'-GCGCGGGGACGUUGGUGUdTdT-3'), as described (24–27).

Preparation of Nuclear and Cytoplasmic Fractions—MCF7 cells (~4 × 10⁵ per 60-mm dish) transfected with the indicated expression vectors (wild-type and mutant forms of MIF) or a MIF-specific siRNA 1 were used for the preparation of nuclear and cytoplasmic fractions for immunoblot analyses, as described previously (22).

Reporter Assays—Luciferase activity was monitored by using a p53-Luc reporter containing eight copies of the p53-responsive element derived from the p21 promoter (kindly provided by Dr. Y.-I. Yeom, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea). MCF7 or U2OS cells were transiently transfected according to the WelFect-Ex™ Plus method with the reporter plasmids, along with the appropriate expression vectors as indicated. Luciferase activity was assessed using a luciferase assay kit (Promega), according to the manufacturer’s instructions.

Ubiquitination Assay—p53-null HCT116 cells were transfected with plasmids encoding p53, wild-type,
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and mutant forms of MIF, MIF-specific siRNAs 1 and 2, and HA-tagged ubiquitin either alone or in combination. The cells were treated with 10 μg/ml MG132 (Calbiochem) for 4 h, harvested, and then washed twice with phosphate-buffered saline (PBS) (pH 7.4), lysed in 200 μl of Tris-buffered saline (pH 7.4) containing 2% SDS, and incubated at 95 °C for 10 min. p53 proteins were immunoprecipitated with an anti-p53 antibody and subjected to SDS-8% PAGE, followed by Western blot analysis with an anti-HA antibody.

Immunodepletion of MIF—HEK293 cell extracts were incubated with 4 μg of anti-MIF antibody for 1 h at 4 °C, and 60 μl of protein A-Sepharose were subsequently added and incubated for an additional 3 h at 4 °C. After immunodepletion twice with an anti-MIF antibody, aliquots of the resulting supernatants were subjected to immunoblotting analysis with an anti-MIF antibody to confirm the immunodepletion of MIF. The MIF-depleted extracts were mixed with recombinant GST alone, GST-MIF(WT), or GST-MIF(C81S) proteins (each 4 μg). The mixtures were then purified on glutathione-Sepharose beads, followed by immunoblot analysis using anti-p53 antibody to determine the complex formation between p53 and MIF.

Apoptosis Assay—The apoptosis assay was performed as described previously (24). MCF7 cells undergoing apoptosis were quantified by using the GFP system. The percentage of apoptotic cells was calculated as the number of GFP-positive cells with apoptotic nuclei divided by the total number of GFP-positive cells visualized under a fluorescence microscope.

FACS Analysis—MCF7 cells transiently expressing wild-type and mutant forms of MIF or MIF-specific siRNAs 1–3, together with transfectants expressing p53 or empty vector alone as controls, were washed with ice-cold PBS and then treated with SFU for 30 h or with doxorubicin for 24 h. The trysonized cells were washed twice with ice-cold PBS and incubated at 37 °C for 30 min with a solution consisting of 50 μg/ml propidium iodide and 1 mg/ml RNase A (Sigma) in 1 mm Tris-HCl (pH 7.5). Flow cytometry was performed on a FACS Calibur-S system (BD Biosciences).

Immunofluorescence Staining—The indirect immunofluorescence method was described previously in detail (22). Specimens were examined under a Leica DMire2 confocal laser scanning microscope (Germany).

RESULTS

MIF Directly Interacts with p53 in Mammalian Cells—To test whether MIF can associate with p53 in cells, we transiently expressed wild-type MIF in 293T cells as a FLAG-tagged fusion protein (FLAG-MIF) or expressed an empty vector (cytomegalovirus) as a control. The interactions of FLAG-MIF with endogenous p53 proteins were analyzed by immunoblotting with an anti-FLAG antibody. MIF was detected in the p53 immunoprecipitate (Fig. 1A). To verify the interaction of endogenous MIF with p53 in vivo, we next performed coimmunoprecipitation experiments using MCF7 and HCT116 cell extracts. Endogenous p53 was immunoprecipitated from cell lysates, and the binding of endogenous MIF was subsequently analyzed using Western blotting with an anti-MIF antibody. Again, MIF was present in the p53 immunoprecipitate (Fig. 1B, left panel). To examine whether MIF could bring down p53 in a reciprocal way, endogenous MIF was immunoprecipitated by anti-MIF antibody, followed by immunoblotting with an anti-p53 antibody. The endogenous...
p53 was also detected in the MIF immunoprecipitate (Fig. 1B, right panel). These data demonstrate that MIF physically interacts with p53 in vivo.

Formation of the MIF-p53 Complex Requires the Cysteine Residue (Cys81) of MIF—To investigate whether the cysteine residues of MIF are necessary for the formation of the MIF-p53 complex, we used siRNAs to knock down the expression of endogenous MIF in HCT116 cells (Fig. 5E, right panel). The results show that knockdown of MIF expression significantly reduces the expression of p53 target genes, suggesting that MIF is required for the formation of the MIF-p53 complex.

**FIGURE 5. Inhibition of p53-mediated transcription by MIF.** A, suppression of p53-mediated transcription by MIF. MCF7 and U2OS cells were transfected with increasing amounts of FLAG-MIF as indicated, together with 0.3 μg of p53-Luc reporter plasmid and 0.1 μg of β-galactosidase internal control, in the presence or absence of SFU (0.38 mM). Luciferase expression normalized to LacZ expression from triplicate samples is presented, and the standard deviations are less than 5%. B, up-regulation of p53-mediated transcription by MIF-specific siRNAs. MCF7, U2OS, p53-null H1299, and p53-null HCT116 cells were transfected with increasing amounts of MIF-specific siRNAs as indicated, in the presence or absence of FLAG-p53. A nonspecific control siRNA (Cont. siRNA) was used to adjust the total RNA amounts. Expression levels of endogenous MIF and β-actin were determined by anti-MIF and anti-β-actin immunoblotting (upper right), respectively.

C, effect of wild-type and mutant forms of MIF on p53-mediated transcription. MCF7 and U2OS cells were transfected with 0.6 μg of p53-Luc reporter together with the indicated expression vectors encoding wild-type and mutant forms of FLAG-MIF, in the presence or absence of FLAG-p53. Luciferase activity was measured 48 h after transfection and normalized to β-galactosidase activity. The standard deviations are less than 5%. The data are representative of at least three independent experiments. D, effect of MIF on the expression of p53 target genes. MCF7 and HCT116 cells transfected with the indicated plasmid vectors expressing a control vector (Vector), wild-type GST-MIF, or GST-MIF(C81S) were lysed and subjected to immunoblot analysis using anti-p53, anti-p21, anti-Mdm2, anti-Bax, and anti-β-actin antibodies. E, modulation of p53 target genes by knockdown of endogenous MIF. HCT116 cells stably expressing pSingle-tTS-shRNA empty vector (Vector), HCT116 cells stably expressing pSingle-tTS-shRNA vector containing MIF-specific shRNA (MIF shRNA), or parental HCT116 cells (Parental) treated with SFU (0.38 mM) were cultured in the presence or absence of 1 μg/ml doxycycline (Dox) for 72 h and subjected to immunoblot analysis using anti-p53, anti-p21, anti-Mdm2, anti-Bax, anti-β-actin, and anti-MIF antibodies (left panel). HCT116, MCF7, and U2OS cells were transiently transfected with 200 nM of control siRNA (Cont.), or MIF-specific siRNAs (#1, #2, and #3) as indicated. Cell lysates were subjected to immunoblot analysis using anti-p53, anti-p21, anti-Mdm2, anti-Bax, anti-β-actin, and anti-MIF antibodies (right panel).
complex, we transiently cotransfected 293T cells with GST-tagged wild-type MIF and its mutants, MIF(C57S), MIF(C60S), and MIF(C81S), together with FLAG-tagged wild-type p53. The expression of the MIF mutants MIF(C57S) and MIF(C60S) did not notably influence the association between MIF and p53, whereas the MIF(C81S) mutant dramatically decreased the complex formation (Fig. 2A). We also used nondenaturing PAGE to analyze the association of purified, recombinant MIF proteins with p53 that was translated in vitro. When the 35S-labeled p53 was incubated in the presence of wild-type MIF and its mutants MIF(C57S) and MIF(C60S), the mobility clearly shifted relative to incubation in the absence of MIF (Fig. 2B, 1st lane versus 2nd to 4th lanes). In contrast, the mobility shift was not observed when 35S-labeled p53 was incubated with β-mercaptoethanol considerably decreased the amount of coprecipitated MIF, whereas H2O2, an oxidant, did not (Fig. 2D), suggesting that the interaction of MIF with p53 in vivo is redox-dependent. These data strongly suggest that the in vivo association of MIF and p53 requires the participation of cysteine residues.

**Determination of the MIF Interaction Domain of p53**—To map the domain(s) within p53 required for its association with MIF, we generated a set of six p53 deletion mutants (Fig. 3A, upper panel), and we examined their ability to interact with MIF using in vitro binding assays in 293T cells. MIF interacted with wild-type p53, p53(44/393), p53(44/387), p53(319/393), and p53(DBD) but not with p53(319/360) and p53(TAD) (Fig. 3A, lower panel). These results indicate that the interaction of MIF(C81S) (Fig. 2B, 5th lane). This was further confirmed by measuring the binding capacity of recombinant MIF(C81S) with endogenous p53 in MIF-depleted extracts. A weak interaction between MIF(C81S) and p53 that was observed in normal cell extracts had completely disappeared in MIF-depleted extracts (Fig. 2C), providing additional evidence of a physical association between MIF and p53 through Cys81. As a control, the efficiency of depletion was shown to be 100% by immunoblotting with an anti-MIF antibody (Fig. 2C, bottom panels). To address the question of whether MIF specifically binds to p53 through Cys81, we also employed an inducible MIF shRNA system to deplete the expression of endogenous MIF in HCT116 cells. A similar result was also observed in a stable system for tetracycline-inducible expression of MIF shRNA (Fig. 2D, right panel). As a control, HCT116 cells stably expressing pSingle-tTS-shRNA vector harboring MIF-specific shRNA (MIF shRNA) showed a doxycycline-dependent RNA interference effect on the endogenous MIF silencing (Fig. 2D, right bottom panel), whereas stable HCT116 cells containing the empty vector alone (Vector Laboratories) showed no effect on the expression of endogenous MIF in the presence or absence of doxycycline (Fig. 2D, left upper and right bottom panels). These observations led us to investigate the effect of the redox status on the formation of the endogenous MIF-p53 complex in cells. The reductants DTT and H2O2, an oxidant, did not (Fig. 2D), suggesting that the interaction of MIF with p53 in vivo is redox-dependent. These data strongly suggest that the in vivo association of MIF and p53 requires the participation of cysteine residues.

**FIGURE 5—continued**
MIF with p53 is mediated via the DNA binding domain (DBD) within residues 113–290. The basic domain within residues 363–393 served as a secondary interaction domain. The central DBD contains all the cysteine residues of p53. Three cysteine residues in murine p53 (Cys173, Cys235, and Cys239) are essential for the suppression of transformation, transactivation, and in vitro DNA binding (28). To examine the involvement of specific cysteines in MIF binding, we initially generated three human p53(DBD) substitution mutants, C176S, C238S, and C242S (Fig. 3B), and examined their ability to interact with MIF. MIF strongly interacted with the wild-type p53(DBD) and the C176S mutant but not with the C242S mutant (Fig. 3C, upper left panel). In addition, cells expressing the C238S mutant formed less of the complex than wild-type p53(DBD). This suggests that Cys238 of p53, like Cys 242, potentially plays a role in the association of p53 with MIF, although to a somewhat lesser extent. To further examine the roles of Cys238 and Cys242 of the DBD domain of p53 in its association with MIF, we generated three p53(DBD) double substitution mutants, C176S/C238S, C176S/C242S, and C238S/C242S, and examined their binding properties in the in vivo binding assay. The expression of C176S/C242S or C238S/C242S dramatically inhibited complex formation between MIF and p53, as compared with the expression of the control p53(DBD) (Fig. 3C, upper right panel). These results suggest that Cys238 of p53, like Cys242, potentially plays a role in the association of p53 with MIF, although to a somewhat lesser extent. To further examine the roles of Cys238 and Cys242 of the DBD domain of p53 in its association with MIF, we generated three p53(DBD) double substitution mutants, C176S/C238S, C176S/C242S, and C238S/C242S, and examined their binding properties in the in vivo binding assay. The expression of C176S/C242S or C238S/C242S dramatically inhibited complex formation between MIF and p53, as compared with the expression of the control p53(DBD) (Fig. 3C, upper right panel). These results suggest that both Cys238 and Cys242 of the DBD domain of p53 are important for its association with MIF; however, Cys242 had a stronger effect on formation of the complex between MIF and p53 than Cys238. We further analyzed whether the Cys81 of MIF and Cys242 (and Cys238) of p53 play a critical role in the association of MIF with p53 using an in vivo binding assay. As expected, expression of MIF(C81S) strongly inhibited the association in the presence of FLAG-C242S, whereas wild-type MIF, MIF(C57S), and MIF(C60S) did not modulate the association of the proteins (Fig. 3C, lower panel). Together, these results strongly suggest that MIF binds to p53 through Cys81 of MIF and Cys242 (and Cys238) of p53.

**MIF-p53 Association Is Regulated by 5FU and Doxorubicin**—5FU is an anti-metabolite that inhibits thymidylate synthase and is important for the apoptotic response (29); it is an important inducer of p53-mediated apoptosis. Doxorubicin induces cell cycle arrest by introducing double-stranded DNA breaks (30). We next examined whether 5FU and doxorubicin can influence MIF-p53 complex formation in cells. Upon 5FU treatment, the association between endogenous MIF and p53 was considerably decreased compared with control cells not treated with 5FU (Fig. 4, left panel). Similarly, doxorubicin treatment resulted in a significant decrease in the association between endogenous MIF and p53 (Fig. 4, right panel). This result was also confirmed by a reciprocal immunoprecipitation experiment in which endogenous MIF was immunoprecipitated by anti-MIF antibody, followed by immunoblotting with an anti-p53 antibody (data not shown). Together, these data indicate that the interaction between MIF and p53 appears to be dependent on p53 stimulation by 5FU or doxorubicin.

**MIF Suppresses p53-mediated Transcription**—To identify the physiological role of the MIF-p53 complex, we first analyzed the effect of MIF on p53-mediated transcription. We transiently transfected MCF7 and U2OS cells with increasing amounts of MIF, together with a p53-Luc reporter plasmid, in the presence or absence of 5FU. The addi- tion of MIF inhibited the p53-mediated transcription in a dose-dependent manner (Fig. 5A), suggesting that MIF is a negative regulator of p53 activity. In contrast, expression of MIF alone, as a control, had no effect on the regulation of p53-mediated transcription. We also examined the effect of MIF-specific siRNAs on p53-mediated transcription in cells. The transfection of the MIF-specific siRNAs resulted in a significant increase of p53-mediated transcription that was proportional to the amount of MIF-specific siRNAs transfected (Fig. 5B, upper panel). A similar trend was also observed in p53-null HCT116 and H1299 cells transfected...
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FIGURE 6. Modulation of p53 stability by MIF. A, stabilization of the p53-Mdm2 complex by MIF. MCF7 and HCT116 cells were transfected with increasing amounts (0.8, 1.6, and 3.2 μg) of the wild-type and mutant forms of MIF. The cells were lysed and then immunoprecipitated with an anti-Mdm2 antibody (IP), and immunoblotted with an anti-p53 antibody to determine the endogenous levels of p53-Mdm2 complex formation (left and right, top panels). Expression levels of Mdm2 and p53 were analyzed by immunoblot using anti-Mdm2 and anti-p53 antibodies (left and right, 2nd and 3rd panels). Expression levels of increasing amounts of wild-type MIF and its mutant (C81S) were confirmed by an anti-FLAG antibody immunoblot (left and right, 4th panels). WB, Western blot. B, downregulation effect of endogenous MIF on p53-Mdm2 complex formation. HCT116 cells stably expressing pSingle-tTS-shRNA empty vector (Vector) and HCT116 cells stably expressing pSingle-tTS-shRNA vector containing MIF-specific shRNA (MIF shRNA) were cultured in the presence or absence of 1 μg/ml doxycycline (Dox) for 72 h. Cell lysates were immunoprecipitated (IP) with preimmune serum (preimm.) or an anti-Mdm2 antibody (α-Mdm2) and subjected to an immunoblot analysis using the indicated antibodies (left panel). HCT116 cells were transfected with the indicated expression vectors for p53 and Mdm2, together with increasing amounts (50 and 100 nM) of the MIF-specific siRNAs (#1 and #2). Cell lysates were immunoprecipitated with an anti-Mdm2 antibody (IP), and immunoblotted with an anti-p53 antibody to determine the level of p53-Mdm2 complex formation (right, top panel). C, MIF forms a ternary complex with p53 and Mdm2 in vivo. MCF7 cells were transiently transfected with the appropriate expression plasmids, and GST fusion proteins were purified on glutathione-Sepharose beads (GST purification), resolved by SDS-PAGE, and visualized with ECL. Formation of a ternary complex containing MIF, p53, and Mdm2 was determined by Western analysis using an anti-FLAG antibody (left, top panel). Cell lysates from MCF7 and U2OS cells were immunoprecipitated with preimmune serum (preimm.) or an anti-Mdm2 antibody (α-Mdm2), and blotted with an anti-MIF antibody (right, top panel). The same blot was stripped and re-probed with an anti-p53 antibody (right, 2nd panel). To measure formation of a ternary complex containing MIF, p53, and Mdm2, D, measurement of the stability of p53 by Western blotting with an anti-p53 antibody. MCF7 and HCT116 cells were transiently transfected with the expression vectors (4 μg each) for an empty vector (Vector), wild-type MIF (MIF(WT)), MIF(C57S), MIF(C60S), and MIF(C81S) or MIF-specific siRNAs 1 and 2. Time intervals indicate the number of minutes after cycloheximide (CHX) treatment (20 μg/ml). E, modulation of p53 ubiquitination by MIF. p53-null HCT116 cells were transfected with vectors expressing HA-tagged ubiquitin (Ub), p53, wild-type and mutant forms of MIF as indicated (each 2 μg), control siRNA, and MIF-specific siRNAs 1 and 2. Cell lysates were immunoprecipitated with an anti-p53 antibody (IP), and immunoblotted with an anti-HA antibody to determine the level of p53 ubiquitination (top panels).

with the MIF-specific siRNA, supporting that the inhibitory effect of MIF on p53-mediated transcription is truly p53-dependent (Fig. 5B, lower panel). To further examine whether the MIF-induced inhibition of p53 transcriptional activity is dependent on its direct interaction with p53, we determined the effect of MIF mutants on p53-mediated transcription in MCF7 and U2OS cells. Expression of MIF(C81S) had little effect on p53-mediated transcription, whereas expression of wild-type MIF, MIF(C57S), and MIF(C60S) substantially decreased p53-mediated transcription to a similar extent in a dose-dependent manner (Fig. 5C). We extended this analysis to investigate the effect of MIF on p53 signaling. Overexpression of MIF decreased the expression of p53 target genes, including p53, p21, and BAX, whose genes are normally up-regulated by p53 signals, in cells tested (Fig. 5D); however, overexpression of MIF(C81S) did not have this effect. To further confirm the negative role of MIF in p53 signaling, we generated a stable system for tetracycline-inducible expression of MIF shRNA in HCT116 cells (MIF shRNA) and analyzed them for p53-induced gene expression. Compared with the expression in control HCT116 cells stably expressing the empty vector alone (Vector Laboratories), knockdown of endogenous MIF by doxycycline treatment significantly increased the expression of p53, p21, MDM2, and BAX (Fig. 5E, left panel). MIF-knockdown cells showed a lower induction of p53 target genes compared with parental HCT116 cells (parental cells) treated with 5FU that induces p53-mediated apoptosis (Fig. 5E, left panel, lane 4 versus lane 5). We observed similar results in HCT116 cells transiently transfected with MIF-specific siRNAs (Fig. 5E, right panel). This inverse correlation between MIF-p53 complex formation and activation of p53 function clearly suggests that MIF physically associates with p53 and negatively regulates p53 signaling.

MIF Stabilizes the Association between p53 and Mdm2—To examine how MIF acts as a negative regulator of p53, we tested the effect of MIF on the endogenous p53-Mdm2 interaction using in vivo binding assays. Coexpression of wild-type MIF signifi-
cantly increased the association between p53 and Mdm2 as compared with untransfected control cells (Fig. 6A, left panel). However, coexpression of the MIF(C81S) mutant had no effect on the formation of the p53-Mdm2 complex (Fig. 6A, right panel). To further examine the negative role of MIF in the regulation of p53 activity, we analyzed tetracycline-inducible MIF shRNA cells (MIF shRNA) for the binding of p53 and Mdm2. As expected, knockdown of endogenous MIF by doxycycline markedly decreased the association between p53 and Mdm2 relative to the association in control MIF shRNA cells untreated with doxycycline (Fig. 6B, left panel). We also performed siRNA experiments in HCT116 cells to confirm the above result. The MIF-specific siRNAs decreased the interaction between p53 and Mdm2 in a dosedependent manner as compared with the control cells not transfected with MIF-specific siRNAs (Fig. 6B, right panel). Based on this, we examined whether MIF, p53, and Mdm2 could form a ternary complex using cotransfection and coimmunoprecipitation experiments. MCF7 cells were transfected with GST-p53, FLAG-Mdm2, and FLAG-MIF. Both Mdm2 and MIF were detected in the coprecipitate only when coexpressed with GST-p53 but not with the control GST alone (Fig. 6C, left panel). A ternary complex formation was also demonstrated by coimmunoprecipitation of MIF, p53, and Mdm2 in MCF7 and U2OS cells. Both MIF and p53 were only present in the Mdm2 immunoprecipitate but not in the control (Fig. 6C, right panel). These results indicate that MIF, p53, and Mdm2 are in a ternary complex in vivo. Furthermore, to confirm the negative role of MIF in the regulation of p53 activity, we measured the stability of p53 using a Western blot analysis in MCF7 and HCT116 cells. Expression of wild-type MIF, MIF(C57S), and MIF(C60S) considerably decreased the stability of p53 as compared with the control (Vector Laboratories), but this effect was not observed in the presence of MIF(C81S), which is unable to bind to p53 (Fig. 6D, left panel). Similar results were also observed in cells transfected with MIF-specific siRNAs (Fig. 6D, right panel). To determine p53 ubiquitination in the presence of wild-type and mutant forms of MIF, we also performed an in vivo ubiquitination assay in which p53-null HCT116 cells were transfected with p53 and HA-tagged ubiquitin (30). In agreement with the results obtained from the analysis of p53 stability (Fig. 6D), coexpression of wild-type MIF, MIF(C57S), and MIF(C60S) induced p53 ubiquitination as compared with the control expressing p53 alone (p53), whereas MIF(C81S) had no effect on ubiquitination of p53 (Fig. 6E, left panel). Consistently, the
knockdown of MIF with MIF-specific siRNAs resulted in a significant decrease in p53 ubiquitination, whereas a control siRNA had no effect (Fig. 6E, right panel).

**MIF Inhibits p53 Nuclear Translocation**—We next examined whether MIF inhibits p53-mediated translocation by affecting p53 nuclear translocation. MCF7 cells were transiently transfected with the indicated expression vectors encoding wild-type and mutant forms of MIF and a MIF-specific siRNA 1 in the presence of p53. Cells were immunostained with anti-p53 or anti-MIF antibodies, followed by an Alexa Fluor-594 anti-mouse secondary antibody (for MIF, green), and analyzed by confocal microscopy. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI). B, confirmation of p53 localization by Western blot analysis. Cytoplasmic and nuclear fractions of MCF7 cells transfected with the indicated expression vectors or a MIF-specific siRNA 1 in the presence of exogenous p53 were analyzed by immunoblot using the indicated antibodies (upper panels, exogenous p53). The localization of endogenous p53 was also determined by immunoblot under the same conditions (lower panels, endogenous p53). Nucleus and cytosol indicate the nuclear and cytoplasmic fractions, respectively. The data are representative of at least three independent experiments.

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with control parental MCF7 cells (Fig. 7A, 5th panel versus 6th panel). We further examined whether MIF affects the p53 nuclear translocation using an immunoblot analysis of cytoplasmic and nuclear fractions of cell extracts transfected with expression vectors encoding wild-type and mutant forms of MIF or a MIF-specific siRNA. p53 mainly accumulated in the nuclear fraction of cells in the presence of exogenous p53, but the distribution shifted mainly to the cytoplasm upon expression of wild-type MIF, MIF(C57S), or MIF(C60S) (Fig. 7B, left panel, exogenous). In addition, as expected, there was a corresponding increase in the accumulation of cytoplasmic p53, consistent with the confocal microscopy data. However, no difference was observed in the subcellular localization of p53 between untransfected control cells and cells expressing MIF(C81S). To confirm that MIF is responsible for modulating the intracellular localization of p53 under physiological conditions, we also performed a Western blot analysis in the absence of exogenous p53 under the same conditions. Similar results were observed for the translocation of endogenous p53 (Fig. 7B, left panel, endogenous). To verify whether the knockdown of endogenous MIF could modify the subcellular localization of p53, MCF7 cells transfected with a MIF-specific siRNA were separated into cytoplasmic and nuclear fractions. Each fraction was analyzed by Western blot analysis. The accumulation of p53 in the nucleus fraction was significantly increased in MIF-knockdown cells compared with control cells not transfected with a MIF-specific siRNA (Fig. 7B, right panel). These results suggest that MIF prevents the nuclear accumulation of p53 by stimulating p53 degradation.

MIF Inhibits p53-induced Apoptosis and Cell Cycle Arrest—To determine whether MIF can regulate p53-driven apoptosis and cell cycle arrest, we first examined the effect of wild-type and mutant forms of MIF or MIF-specific siRNAs on p53-induced apoptosis using a GFP assay system in MCF7 cells. Approximately 41% of the MCF7 cells not transfected with MIF were apoptotic following 5FU treatment (Fig. 8A). Transfection with wild-type MIF, MIF(C57S), or MIF(C60S) suppressed apoptosis by about 49% relative to control MCF7 cells untransfected with MIF. However, the inhibitory effect of MIF on p53-induced apoptosis was not detected in cells expressing
MIF(C81S). The extent of apoptosis among transfected cells was also confirmed by a flow cytometry analysis. Compared with cells expressing p53 alone, cells expressing wild-type MIF, MIF(C57S), or MIF(C60S) had a lower percentage of cells in sub-G1 phase after 5FU treatment (Fig. 8B). However, MIF(C81S) did not influence the percentage of cells in sub-G1 phase, again supporting the importance of the direct interaction between MIF and p53 in the regulation of p53 signaling. Furthermore, the knockdown of endogenous MIF with MIF-specific siRNAs resulted in a significant increase in p53-induced apoptosis (Fig. 8A and B).

We extended our analysis to examine whether MIF can affect p53-induced cell cycle arrest. Under the same conditions, p53-induced G0/G1 arrest was lower in cells expressing wild-type MIF, MIF(C57S), or MIF(C60S) than in control cells expressing p53 alone (Fig. 8C), but expression of MIF(C81S) had little or no effect on p53-induced G0/G1 arrest. In addition, the opposite trend was observed in MIF-knockdown cells compared with control cells expressing p53 alone (Fig. 8C, 2nd lane versus 7th to 9th lanes). Together, these results suggest that Cys81 of MIF is essential for the regulation of p53-induced apoptosis and cell cycle arrest, which is probably achieved by mediating the physical interaction of MIF with p53.

**DISCUSSION**

This study demonstrates that MIF interacts with p53 in vivo, and that the MIF-p53 interaction depends on the redox status and cysteine residues present in each of the proteins. In addition, we found that the direct association of MIF with p53 is critical to the ability of MIF to regulate the p53-dependent pathway.

The molecular mechanism by which MIF inhibits p53 activity had not been known previously. Several recent studies suggested that MIF is

**FIGURE 8. Effect of MIF on p53-mediated apoptosis and cell cycle arrest.**

A, effect of MIF on p53-mediated apoptosis. MCF7 cells were transiently transfected with the indicated expression vectors (2 μg each) for wild-type MIF (MIF(WT)), MIF(C57S), MIF(C60S), and MIF(C81S) or MIF-specific siRNAs (200 nM each), together with 2 μg of p53 and 1 μg of GFP, in the presence (black bars) or absence (white bars) of 5FU (0.38 mM). Apoptotic cell death was determined using the GFP expression system, as described previously (24). GFP-positive cells were examined with a fluorescence microscope to detect apoptotic nuclei. The data shown are the mean ± S.D. of duplicate assays and are representative of at least four independent experiments. B and C, effect of MIF on cell cycle distribution. MCF7 cells transfected with wild-type and mutant forms of MIF (3 μg each) or MIF-specific siRNAs (200 nM each), together with p53 (1 μg), were treated with 5FU (0.38 mM) for 30 h or doxorubicin (100 ng/ml) for 24 h, and the sub-G1 DNA content was analyzed by FACSscan. Apoptotic cells in each untreated or 5FU-treated sample are shown as the sub-G1 population (B). The indicated percentages represent the G0/G1 (white bars) and G2/M (black bars) arrest in response to doxorubicin (C). As a control, MCF7 cells expressing pcDNA-His empty vector that were not transfected with p53 (Vector) are shown. These experiments were independently performed at least three times with similar results.
a potent modulator of the p53-dependent pathways, which are stimulated by oncogenic signaling (31, 33). We previously demonstrated that cysteine residues of MIF play an important role in the association of proliferation-associated gene, an antioxidant, suggesting that MIF may use its cysteine residues to heterodimerize with other cellular proteins (9). To address this, we investigated the roles of individual cysteine residues of MIF in the direct interaction with p53 using in vivo binding assays of transiently expressed wild-type and mutant forms of MIF and p53 and demonstrated that MIF associates with p53 through Cys\(^{81}\) of MIF and Cys\(^{242}\) (and Cys\(^{238}\)) of p53 in mammalian cells (Figs. 2 and 3). In addition, we observed that the Cys to Ser mutations of MIF do not alter the normal functions of this protein, including MIF-mediated self-association and proliferation of quiescent NIH 3T3 fibroblasts (data not shown). To gain more insight into the roles of cysteine residues in the MIF-p53 association, we also examined the in vivo binding of MIF and p53 under various redox conditions. As shown in Fig. 2E, in reducing conditions the association was remarkably decreased in a dose-dependent manner, indicating that the in vivo association of MIF and p53 requires the participation of cysteine residues. On the other hand, previous studies showed that JAB1 specifically interacts with both p53 and MIF (12, 34), suggesting that JAB1 becomes a bridge connecting p53 and MIF. However, our studies using JAB1-depleted extracts provide evidence that MIF directly interacts with p53 in the absence of JAB1 even though we cannot exclude the possibility that MIF indirectly interacts with p53 through JAB1 (data not shown).

The inhibition of p53 activity by MIF, and the interaction of MIF with p53, prompted us to investigate whether MIF affects p53 stability through Mdm2, a known negative regulator of p53. An in vivo binding assay was performed in MCF7 and HCT116 cells transfected with wild-type MIF or MIF(C81S) mutant (Fig. 6A). When wild-type MIF was expressed in these cells, it stabilized the association between p53 and Mdm2 in a dose-dependent manner. However, when MIF(C81S), which is unable to bind with p53, was expressed, it did not alter the level of the association. In addition, MIF ubiquitinated p53 as expected in an in vivo ubiquitination assay (Fig. 6D). These data suggest that MIF inhibits p53 function through the stabilization of the p53-Mdm2 complex.

Previous studies had shown that MIF contains the conserved cysteine sequence motif (Cys-X-X-Cys) that is important for the oxidoreductase and macrophage-activating activities of MIF. Of the Cys-to-Ser substitution mutants of MIF, the MIF(C60S) mutant did not exhibit the oxidoreductase and macrophage-activating activities of MIF (11), whereas the MIF(C81S) mutant was active in both activities of MIF. However, MIF(C81S) exhibited a somewhat different secondary structure than the wild-type and other mutant forms of MIF, indicating that MIF(C81S) could mediate a conformational effect. This would also imply that the effect of MIF(C81S) on the physical association between MIF and p53 we saw in this study could have been due to conformational changes. However, the experiments demonstrating the importance of cysteine residues, especially Cys\(^{242}\) (or Cys\(^{238}\)) of p53, in the association of MIF with p53 (Fig. 3C) support a specific role for the cysteine residue, rather than a more general conformational change. In addition, we do not rule out the possibility that interactions other than those occurring through cysteine residues can occur in the association between MIF and p53, because the basic domain of p53 was also involved in the association as a second interaction domain, although to a lesser extent (Fig. 3A). In conclusion, our studies provide evidence that the direct interaction between MIF and p53 through cysteine residues is critical for the modulation of the p53-dependent pathway by MIF. Moreover, examination of the molecular mechanism of the MIF-mediated inhibition of p53 function, including the Mdm2-mediated degradation of p53, is important to understanding how MIF inhibits p53 function and provides insight into the mechanism by which chronic inflammation is associated with tumorigenesis.

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