Histone Arg Modifications and p53 Regulate the Expression of OKL38, a Mediator of Apoptosis

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Protein Arg methyltransferases function as coactivators of the tumor suppressor p53 to regulate gene expression. Peptidylarginine deiminase 4 (PAD4/PADI4) counteracts the functions of protein Arg methyltransferases in gene regulation by demethylation and demethylimination. Here we show that the expression of a tumor suppressor gene, OKL38, is activated by the inhibition of PAD4 or the activation of p53 following DNA damage. Chromatin immunoprecipitation assays showed a dynamic change of p53 and PAD4 occupancy and histone Arg modification at the OKL38 promoter during DNA damage, suggesting a direct role of PAD4 and p53 in the expression of OKL38. Furthermore, we found that OKL38 induces apoptosis through localization to mitochondria and induction of cytochrome c release. Together, our studies identify OKL38 as a novel p53 target gene that is regulated by PAD4 and plays a role in apoptosis.

Post-translational histone modifications play pivotal roles in chromatin-templated nuclear events, such as transcription and DNA damage repair (1–3). Histone Arg methylation catalyzed by members of the protein Arg methyltransferase family correlates with transcriptional activation of β-globin, nuclear receptor, and p53 target genes (4–9). Peptidylarginine deiminases (PADs)2 are a family of enzymes previously known to convert protein Arg residues to citrulline (Cit, a nonconventional amino acid in proteins) (10–12). In searching for enzymes that reverse histone Arg methylation, as well as a decrease in PAD4 association and histone Arg modifi-

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Treatments with Cl-amidine, Doxycycline, Doxorubicin, and siRNAs—These procedures are described in detail in the supplemental material.

Plasmid Construction, Antibody Generation, Quantitative RT-PCR, and Chromatin Immunoprecipitation—Details about these procedures are described in the supplemental material.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay (EMSA) was performed according to a method described previously (28), with some modifications. Oligonu-
cleotides called BB9, corresponding to the DNA binding consensus sequence specific for human p53 (29), 5‘-TGTCCGGGCATGTCGGGGCATGTCCGGGGCATGT-3’, the potential p53-binding sites derived from OKL38 Oligo-1, 5‘-GTAGCTGGGTTACAGTGACCCCATGCGCCCTGCC-3’, and Oligo-2, 5‘-TGCTGGCCCTTAACAGTATCGGCCTTTGTC-3’, were used in these assays. Complementary oligonucleotides were annealed by heat treatment at 95 °C for 5 min and then kept at room temperature for 20 min. Oligonucleotides were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (New England Biolabs). FLAG-p53 fusion protein was purified from BL21. EMSA was performed in a two-step procedure. In the first step, p53 was activated with monoclonal antibody, PAB421, in the DNA binding buffer (10 mM HEPES, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 18% glycerol, 0.05% Nonidet P-40, 50 mM dithiothreitol, 4 mM spermidine, 11 μg/ml of poly(dI-dC) for 30 min at room temperature. In the second step, 0.3 ng of labeled DNA probe was added, and a second incubation for 30 min at room temperature was performed. Reaction products were loaded onto a 4% polyacrylamide gel containing TBE. Electrophoresis was performed for 1.5 h at 100 V. Gels were dried and exposed to x-ray film.

analyses were performed using the FC500 flow cytometer. At least 10,000 cells were analyzed.

Nuclear Extract Preparations—Procedure is described in detail in the supplemental material.

RESULTS

PAD4 Regulates the Expression of the OKL38 Gene—We and others (13, 15) have previously studied the role of PAD4 in gene regulation using breast cancer MCF-7 cells. To analyze the effects of PAD4 inhibition by Cl-amidine on global gene expression, microarray analyses were performed using MCF-7 cells treated with 200 μM Cl-amidine for 24 h. One of the genes activated by Cl-amidine, pregnancy-induced growth inhibitor/OKL38/BDGI, was previously reported to play a role in cell growth inhibition and tumorigenesis (26, 27, 30). To further test the induction of OKL38 by Cl-amidine, mRNA from MCF-7 cells treated with or without Cl-amidine was analyzed by quantitative reverse transcription-PCR (qRT-PCR). After normalizing mRNA levels to GAPDH, an ~4.5-fold induction of OKL38 was detected (Fig. 1A). Additionally, qRT-PCR assays found that the expression of OKL38 was induced >4-fold after Cl-amidine treatment in the osteosarcoma U2OS cells (Fig. 1B),
suggesting that CI-amidine can induce OKL38 expression in multiple cell types. Furthermore, qRT-PCR analyses showed that OKL38 was activated by CI-amidine in a dosage-dependent manner (Fig. 1C).

To test the change of OKL38 protein levels after CI-amidine treatment, we generated a rabbit polyclonal antibody against His6-OKL38 (residues 1–477, NCBI protein accession number AAP14664) expressed in and purified from Escherichia coli (supplemental Fig. S1A). The antibody was affinity-purified, and the specificity of the antibody was confirmed by antigen competition experiments (supplemental Fig. S1B). A band with a molecular mass of ~52 kDa, corresponding to the predicted molecular weight of the 477-amino acid OKL38 protein, was detected after CI-amidine treatment in MCF-7 and U2OS cells (Fig. 1D). In contrast, the amount of OKL38 protein was hardly detectable before CI-amidine treatment.

As an alternative to PAD4 inhibition by CI-amidine, we used siRNAs to deplete PAD4 in U2OS cells. The siRNA treatment resulted in >50% decrease in the levels of PAD4 protein (Fig. 1E). qRT-PCR assays indicated that the expression of OKL38 was elevated ~2.5-fold after PAD4 depletion (Fig. 1F). These results suggest a repressive role of PAD4 in the expression of OKL38.

Because PAD4 can deiminate and demethylminate histones, we postulated that histone Arg citrullination and methylation might be altered at the OKL38 promoter after PAD4 inhibition by CI-amidine. To test this possibility, ChIP assays were performed to analyze PAD4, histone H3 Arg-17 methylation (H3R17Me), and histone H3 citrullination (CitH3) at the OKL38 promoter in U2OS cells after CI-amidine treatment. The CitH3 antibody was made against an H3 N-terminal peptide (residues 1–20) containing three citrulline residues (Cit2, -8, and -17) (13). ChIP analyses showed that the amount of PAD4 was not significantly altered at the OKL38 promoter (Fig. 1G). Consistent with PAD4 inhibition by CI-amidine, histone H3R17Me was increased whereas histone H3 citrullination was decreased at the OKL38 promoter after CI-amidine treatment (Fig. 1G). As a control, we found that PAD4 did not associate with the GAPDH promoter, and the amount of histone H3 Arg-17 methylation and citrullination was hardly detected (Fig. 1G, see also representative gel images in supplemental Fig. S2). These results correlate the decrease of histone citrullination and the increase of histone Arg methylation with the induction of OKL38 by CI-amidine.

OKL38 Is a Putative p53 Target Gene—Our results suggest that PAD4 is involved in the regulation of OKL38. How PAD4 is targeted to the OKL38 promoter to regulate transcription is unknown. Because our recent work found that PAD4 interacts with p53 and regulates the expression of the p53 target gene p21 (17), we postulated that p53 might function as a transcription factor to recruit PAD4 to the OKL38 promoter. To analyze whether p53 regulates the expression of OKL38, the p53-/- lung carcinoma H1299 cells were transiently transfected with a FLAG-p53 expressing plasmid. At 48 h after transfection, a significant increase of OKL38 mRNA was detected by qRT-PCR (Fig. 2A). To further investigate whether the transcriptional activation function of p53 is important for OKL38 induction, we analyzed the expression of OKL38 in Tet-On osteosarcoma Saos-2 cells that express the wild type p53 or an inactive p53R175H mutant that has a defect in activating gene expression (31, 32). Western blot experiments indicated that the wild type p53 and the p53R175H mutant were expressed at comparable levels after treatment with doxycycline for 12 h (supplemental Fig. S3). RT-PCR assays showed that OKL38 mRNA was increased in Saos-2 cells after the expression of the wild type p53 (Fig. 2B, lanes 1 and 2), but not in cells after the expression of the p53R175H mutant (Fig. 2B, lanes 3 and 4), suggesting that the transcriptional activation function of p53 is required for the induction of OKL38. In controls, the p53 target gene p21 was also only induced by the wild type p53, whereas the GAPDH expression was unaltered (Fig. 2B). Taken together, the above results suggest that the expression of OKL38 can be activated by p53.
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We envisioned two scenarios for the activation of OKL38 by p53, either p53 directly binds to the OKL38 promoter to activate transcription or p53 indirectly activates OKL38 via other protein factors. To analyze whether OKL38 is a downstream target gene of p53, bioinformatics analysis of the OKL38 promoter was first performed using the PROMO program (supplemental Fig. S4A). The PROMO program predicted several putative p53-binding sites in the OKL38 promoter. Two DNA oligos, Oligo-1 and Oligo-2, containing the predicted p53-binding site at −720 and −40 bp, respectively (Fig. 2C, oligo sequences shown in supplemental Fig. S4B), were synthesized. The ability of these oligos to bind FLAG-His6-p53 purified from E. coli (supplemental Fig. S4C) was tested in EMSA. The BB9 oligo, containing a known p53-binding consensus sequence (28), was used as a positive control. Oligo-1 showed p53 binding activity in EMSAs (Fig. 2D, lane 4) but not Oligo-2 (lane 6). Compared with that of BB9 (Fig. 2D, lane 2), the binding of Oligo-1 to p53 was slightly weaker. To further test the binding specificity, we performed EMSAs with the competition of excessive amounts of unlabeled DNA oligos. As shown in Fig. 2E, the binding of p53 to the radioactive BB9 oligo was competed by an excess amount of the unlabeled BB9 oligo (lane 3) or Oligo-1 (lane 4) but not by Oligo-2 (lane 5). Similarly, the binding of the radioactive Oligo-1 to p53 was competed by an excess amount of the unlabeled BB9 (Fig. 2E, lane 8) or Oligo-1 (lane 9) but not by Oligo-2 (lane 10). In sum, the −720-bp region of the OKL38 promoter contains a p53-binding site.

To further analyze whether the −720-bp region of the OKL38 promoter is associated with p53, ChIP was performed in p53−/− H1299 cells with or without transient transfection of a p53-expressing plasmid. p53 binding to the OKL38 promoter was detected only after the expression of p53 (Fig. 2F, lane 3, bottom panel). In control, the GAPDH promoter was not immunoprecipitated by the p53 antibody regardless of the p53 expression status (Fig. 2G, lane 3). The association of p53 to the OKL38 was also detected in MCF-7 cells after DNA damage (see Fig. 3).

OKL38 Is Induced by DNA Damage in a p53-dependent Manner and Is Regulated by Histone Arg Modifications—DNA damage activates p53 and induces the expression of p53 target genes (19, 33). To further analyze whether OKL38 is downstream of the p53 pathway, we treated MCF-7 cells with doxorubicin, a DNA damage-inducing reagent. After normalizing the OKL38 signals to that of the GAPDH, the expression of OKL38 was increased ~2.6 and ~3.5-fold at 6 and 12 h after doxorubicin treatment, respectively (Fig. 3A), indicating that OKL38 is induced by DNA damage. An induction of OKL38 expression was also observed in U2OS cells after doxorubicin treatment (Fig. 3B). Next, to analyze whether the induction of OKL38 by DNA damage is dependent on p53, homotypic p53+/+ and p53−/− HCT116 cells were treated with doxorubicin. qRT-PCR analyses showed that OKL38 expression in p53+/+ HCT116 cells was increased ~2- and ~3.6-fold at 6 and 12 h after doxorubicin treatment, respectively (Fig. 3C, gray bars). In contrast, the levels in OKL38 expression in p53−/− HCT116 cells remained unaltered after doxorubicin treatment (Fig. 3C, dark bars), suggesting that OKL38 is induced by DNA damage in a p53-dependent manner.

Histone Arg methylation has been correlated with the activation of p53 target genes (5), whereas histone deamination and demethylation catalyzed by PAD4 have been correlated with transcriptional repression (13, 15). To analyze whether histone Arg modifications by PAD4 are involved in the tran-
The transcriptional regulation of OKL38 during DNA damage, ChIP experiments were performed to analyze p53, PAD4, and histone Arg modifications at the OKL38 promoter at different time points after doxorubicin treatment in MCF-7 cells. We found that the amount of p53 increased on the OKL38 promoter at 2- and 6-h time points after doxorubicin treatment (Fig. 3D), suggesting that p53 associates with the OKL38 promoter to regulate transcription after DNA damage. In contrast, the amount of PAD4 detected on the OKL38 promoter decreased at 2 and 6 h (Fig. 3D), suggesting that PAD4 is released from the OKL38 promoter during transcriptional activation. To test the change of histone Arg modifications at the OKL38 promoter, we performed ChIP assays with H3 Arg-17 methylation (H3R17Me) and H3 citrullination (CitH3) antibodies. H3R17Me gradually increased on the OKL38 promoter after DNA damage, suggesting a positive role of histone Arg methylation during the process of OKL38 induction (Fig. 3D). In contrast, the level of CitH3 on the OKL38 promoter significantly decreased at 2 and 6 h (Fig. 3D). This decrease in histone citrullination was correlated with the dissociation of PAD4 from the OKL38 promoter, which is consistent with the role of PAD4 in histone citrullination. As a control, GAPDH promoter was not associated with p53, PAD4, or dynamic histone Arg modifications after DNA damage. Representative gel images of ChIP assays are shown in supplemental Fig. S4D.

**OKL38 Overexpression Leads to Apoptosis in U2OS Cells**—The expression of OKL38 has been found to be repressed in 60–70% of human kidney and liver cancers (34, 35). Consistent with the idea that the loss of OKL38 expression is beneficial to cancer cell growth (27, 30), we found that the endogenous OKL38 protein in U2OS cells and MCF-7 cells was hard to detect (Fig. 1D). To analyze the function of OKL38, we used a plasmid vector to express FLAG-OKL38 in U2OS cells (Fig. 4A). FLAG-OKL38 expression in U2OS cells induced nuclear morphology changes characteristic of apoptotic cells (Fig. 4, B and C). To analyze whether OKL38 overexpression induced phosphatidylserine externalization, a hallmark of apoptosis, we performed annexin V staining in U2OS cells 24 h after transient transfection. In control cells transfected with the pIRES vector alone, 7.1% of U2OS cells were positive for the staining of annexin V (Fig. 4D), with 3.4% of cells positive for annexin V only (indication of early apoptotic cells) and 3.6% of cells positive for both annexin V and propidium iodide (measuring cell membrane permeability) staining (Fig. 4E). These low percentages of apoptotic cells likely reflect the cytotoxicity of the transfection reagent. In contrast, when the FLAG-OKL38/pIRES plasmid was transfected into U2OS cells, the annexin V positive cells were increased to 14.5% (Fig. 4F), with 12.0% of cells positive for annexin V and 2.4% of cells positive for both annexin V and propidium iodide (Fig. 4G). Because less than 30% of the U2OS cells are routinely transfected in our procedures, the 14.5% of apoptotic cells detected above indicated that a significant percentage of FLAG-OKL38-expressing cells underwent apoptosis.

**Translocation of Ectopically Expressed OKL38 to Mitochondria**—Although OKL38 overexpression was found to decrease cell growth and increase apoptosis (27, 30), the mechanism by which OKL38 overexpression leads to cell apoptosis is unclear. To address this question, we first analyzed the subcellular distribution of FLAG-OKL38 after ectopic expression in U2OS cells. After transient transfection, FLAG-OKL38 was
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Figure 5. Subcellular localization of OKL38 to nucleus and mitochondria in U2OS cells. A, nuclear localization of FLAG-OKL38 staining was detected in U2OS cells at 24 h after transfection. B, spotted cytoplasmic FLAG-OKL38 staining was also detected in U2OS cells at 24 h after transfection. C, proteins from U2OS cells expressing FLAG-OKL38 were separated into nuclear and cytoplasmic fractions. The presence of FLAG-OKL38, NPM, and tubulin in the nuclear or cytosolic fractions was analyzed by Western blot. D, percentages of cells with mainly cytoplasmic or nuclear OKL38 were analyzed under the microscope. Over 200 U2OS cells scored from three independent experiments. However, OKL38 staining did not colocalize with lysosomes (data not shown).

The inducible expression of OKL38 by Cl-amidine or doxorubicin prompted us to analyze whether endogenous OKL38 is localized to mitochondria after induction. Immunostaining showed OKL38 localization to mitochondria in U2OS cells after Cl-amidine treatment (Fig. 5E) or doxorubicin (G) treatment. The localization of endogenous OKL38 to mitochondria was detected in ~7.8% (25/320) and ~13.5% (44/325) of U2OS cells at 24 and 48 h after Cl-amidine treatment, respectively, but not before treatment. Similarly, ~8.4% (18/213) and ~24.7% (65/
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420) of U2OS cells showed mitochondria localization of OKL38 at 24 and 48 h after doxorubicin treatment, respectively. These results indicate that endogenous OKL38 localizes to mitochondria after induction.

Previously, it was found that OKL38 overexpression inhibited cell growth in MCF-7 cells but not in HeLa cells (27). To test whether the mitochondria localization of OKL38 is associated with its proapoptotic function, we analyzed the subcellular localization of OKL38 in HeLa cells. Stable FLAG-OKL38 expression was established in HeLa cells by retrovirus infection (supplemental Fig. S7). OKL38 was detected in the cytoplasm and the nucleus of HeLa cells (supplemental Fig. S7) but not in mitochondria. The lack of mitochondria localization of OKL38 in HeLa cells suggests that mitochondria localization is important for the cell growth inhibition function of OKL38.

OKL38 Overexpression Led to Mitochondrial Morphology Change and Cytochrome c Release—Many apoptosis-inducing signals converge to mitochondria, leading to changes in mitochondria morphology and the release of cytochrome c (24, 38, 39). To analyze the effects of FLAG-OKL38 expression on mitochondrial morphology and cytochrome c localization, we performed triple labeling to detect mitochondria with MitoTracker, cytochrome c with a mouse monoclonal antibody, and OKL38 with a rabbit polyclonal antibody in U2OS cells. As shown in Fig. 6A, in cells without FLAG-OKL38 expression, filamentous mitochondria structures were stained by both MitoTracker and cytochrome c, and overlapping of MitoTracker and cytochrome c was observed. After the expression of FLAG-OKL38, a formation of large and spotted mitochondria structures was observed by the Mito-Tracker staining (Fig. 6, B and C). Interestingly, overexpression of Bak, a proapoptotic member of the Bcl-2 family of proteins, also caused the formation of speckled mitochondria structures (23). In normally growing cells, cytochrome c is localized to mitochondria, whereas the release of cytochrome c leads to the activation of caspases and apoptosis (24, 38). In ~19% (32/169) of U2OS cells with speckled OKL38 staining in the cytoplasm, a delocalization of cytochrome c from mitochondria was observed (Fig. 6, B and C, denoted by arrows). Taken together, the above results found that OKL38 induced apoptosis in U2OS cells by affecting mitochondria morphology and cytochrome c localization.

DISCUSSION

In this study, we showed that 1) the expression of OKL38 is regulated by p53 and PAD4, and 2) increased OKL38 can induce apoptosis. Our studies suggest that before DNA damage, OKL38 expression is repressed, and its promoter is associated with low levels of p53 and high levels of PAD4. After DNA damage, PAD4 decreases and p53 increases at the OKL38 promoter to activate the expression of OKL38. Upon elevated expression, OKL38 can translocate to mitochondria leading to the change of mitochondria morphology and apoptosis. We show here that inhibition of the histone-modifying enzyme PAD4 by Cl-amidine activates the expression of tumor suppressor gene OKL38. The importance of histone modifications in the control of gene expression during normal and cancerous cell growth has been recognized in recent years (40–42). A paradigm of epigenetic histone modifications in tumorigenesis is emerging, i.e. epigenetic silencing of tumor suppressor genes or activation of oncogenes mediated by histone modifications and DNA methylation participates in the early stage of tumorigenesis.

We have previously shown that PAD4 represses gene expression by regulating Arg methylation and citrullination in histones (15). PAD4 regulates gene expression by counteracting the functions of protein Arg methyltransferases via two possible mechanisms, by deimination to prevent subsequent Arg methylation or by demethylimination to decrease the level of histone Arg methylation (13). Here we show that after inhibition of PAD4 by Cl-amidine to activate OKL38 expression, histone Arg methylation increased whereas histone citrullination decreased at the OKL38 promoter. Additionally, upon DNA damage to activate the expression of OKL38, PAD4 association and histone citrullination at the
OKL38 promoter was decreased with a concomitant increase in histone Arg methylation at the OKL38 promoter. These results suggest that histone Arg modifications play a dynamic role in the regulation of OKL38. We also found that OKL38 is a p53 target gene that is inducible by DNA damage. The expression of wild type p53 but not the p53R175H mutant was sufficient to induce the expression of OKL38. A p53-binding site in the OKL38 promoter was identified by both EMSA and ChIP experiments. The DNA damage reagent doxorubicin increased OKL38 expression in a p53-dependent manner in the p53+/+ but not p53−/− colon cancer HCT116 cells. Furthermore, upon OKL38 activation by doxorubicin, the levels of p53 were increased at the OKL38 promoter, suggesting that p53 plays a positive role in the activation of OKL38 after DNA damage. A recent study has shown that the expression of OKL38 is induced by oxidative stress (43), suggesting that OKL38 can be regulated by multiple extracellular signals.

OKL38 also seems to play an important role in apoptosis. Previous studies have found that the overexpression of OKL38 induced cell death in A498 (kidney cancer), buffalo rat liver, and MCF-7 (breast cancer) cells (27, 34, 35). However, the mechanisms by which OKL38 overexpression induced apoptosis remained unclear. We found that the translocation of OKL38 to mitochondria eventually led to mitochondrial morphology changes, cytochrome c release, and cell death in U2OS cells. Interestingly, upon overexpression, OKL38 was not targeted to mitochondria in HeLa cells and did not induce cell growth inhibition or cell death. Thus, our studies of the mitochondrial localization of OKL38 may offer a molecular mechanism by which OKL38 induces cell death.

The role of p53 in gene regulation has been extensively studied. Following activation, p53 turns on the expression of a class of proapoptotic genes, such as BAX, NOXA, and PUMA, which encode effector proteins that regulate the permeability of mitochondria outer membrane and induce cytochrome c release (18, 19). Because OKL38 induces apoptosis and localizes to mitochondria and regulates cytochrome c release, OKL38 likely belongs to the proapoptotic class of the p53 target genes. The expression of OKL38 is normally induced during pregnancy and lactation in the rat mammary gland, which was proposed to contribute to the cell growth arrest and terminal cell differentiation (30). The loss or decrease of OKL38 protein has been reported in over 60% of hepatocellular carcinoma (26), suggesting that OKL38 may function as a tumor suppressor. How cancer cells gained growth advantage after the loss of OKL38 expression as well as how OKL38 induces cytochrome c release awaits future investigation.

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