PTEN Regulates Mdm2 Expression through the P1 Promoter*

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MDM2 is an oncprotein that controls tumorigenesis through both p53-dependent and -independent mechanisms. Mdm2 mRNA level is transcriptionally regulated by p53 in response to stress such as DNA damage, and its protein level and subcellular localization are post-translationally modulated by the AKT serine/threonine kinase. Previous studies showed that PTEN, a dual specificity phosphatase that antagonizes phosphatidylinositol 3-kinase/AKT signaling, is capable of blocking MDM2 nuclear translocation and destabilizing the MDM2 protein. Results from our current study demonstrate an additional role for PTEN in regulating MDM2 functions; PTEN modulates Mdm2 transcription and isoform selection by negatively regulating its P1 promoter. In Pten-null cell lines and prostate cancer tissues, Mdm2 P1 promoter activity is up-regulated, resulting in increased L-Mdm2 expression and enhanced p90MDM2 isoform production. Furthermore, PTEN controls Mdm2 P1 promoter activity through its lipid phosphatase activity, independent of p53. Thus, our results provide a novel mechanism for PTEN in controlling MDM2 oncprotein functions.

The murine double minute 2 (Mdm2) was originally cloned as an amplified gene present on double minute chromosomes in the tumorigenic 3T3DM murine cell line (1). The human homolog, HDM2, mapping to human chromosome 12q13–14, is overexpressed in over 30% of soft tissue sarcomas due to amplification (2, 3). HDM2 expression is controlled by two different promoters (6–8), leading to alternatively spliced transcripts that differ in their 5’-untranslated regions. Transcription from the first promoter P1 is independent of p53 and yields mRNA (L-HDM2) with exon 2 spliced out. Conversely, transcription from the second promoter P2 is p53-dependent. p53 binds to the two p53-responsive elements in the intron 1 and gives rise to a transcript (S-HDM2) lacking exon 1 but containing exon 2. Multiple gene products of MDM2 have been identified in mammalian cells. The full-length p90MDM2 oncprotein binds to and inactivates the p53 tumor suppressor protein whereas a short p76MDM2 isoform that lacks the first 49 amino acids of p90MDM2 cannot bind p53 (4). A recent study suggests that the ratio of the two MDM2 protein products, p90MDM2 to p76MDM2, is determined by the relative abundance of L-Mdm2 mRNA. The L-Mdm2 predominantly gives rise to p90MDM2 whereas p76MDM2 appears to be the product of translational initiation at codon 50 (AUG) in exon 4 (5).

One of the major biological functions of MDM2 is to regulate p53 level and activity. The p53 tumor suppressor is a short-lived and non-abundant protein in normal cells (9, 10). p53 functions as a transcription factor that up-regulates gene products necessary for cell cycle arrest and apoptosis in response to cell stress such as DNA damage (9). MDM2 regulates p53 activity by at least two mechanisms; MDM2 protein binds p53 in the nucleus and inhibits its transcriptional activity (11–13), and the MDM2-p53 complex shuttles from the nucleus to the cytoplasm (14–16) where MDM2 serves as an E3 ubiquitin ligase and targets p53 to proteasomal degradation (17–19), shortening the half-life of p53 (11–19). Thus, p53 and MDM2 form an autoregulatory feedback loop in which p53 positively regulates MDM2 expression and MDM2 negatively regulates p53 protein level and activity (7). Overexpression of HDM2 has been observed in many primary human cancers. Increasing evidence suggests that, besides regulating p53 levels and functions, MDM2 can also participate in tumorigenesis independent of p53 (31, 33).

PTEN1 tumor suppressor protein is a dual-specificity phosphatase that recognizes both lipid and peptide substrates (21). PTEN dephosphorylates phosphatidylinositol (3,4,5)P3, the product of phosphatidylinositol 3-kinase (PI3K). PTEN mutants that retain protein-tyrosine phosphatase activity but lose the ability to dephosphorylate phosphatidylinositol (3,4,5)P3 are found in tumors, indicating that the lipid phosphatase activity of PTEN is required for its tumor suppressor activity (22, 23). PTEN has been shown to physically interact with p53 and prevent its degradation by excluding a portion of the p53 from the p53-MDM2 complex (24, 29). The PTEN-p53 complex also enhances p53 DNA binding and transcriptional activity (24). Additionally, PTEN inhibits AKT, a key kinase responsible for phosphorylation of MDM2 and its subsequent nuclear translocation (25), destabilizes MDM2, and augments both the level and function of p53 (25–28). In this study, we provided a novel mechanism by which PTEN regulates Mdm2 expression. Our finding suggests that PTEN regulates Mdm2 transcription by suppressing its P1 promoter. PTEN exerts this function indirectly by modulating the activity of unknown transcription factor(s) through its lipid phosphatase activity. Thus, besides the aforementioned mechanisms, PTEN can also up-regulate p53 level and activity by down-regulating Mdm2 transcription and controlling
MDM2 isoform expression. Additionally, PTEN may have a role in suppressing MDM2-mediated tumorigenesis independent of p53 activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—MEF cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone), 100 units/ml penicillin and streptomycin (Invitrogen). Cell transfection was performed using LipofectAMINE (Invitrogen). Cells were lysed 24 h post-transfection for protein and luciferase activity measurements. 

**Plasmid Constructs**—The various 5’-truncated Mdm2 luciferase reporter gene plasmids were created by PCR from mouse genomic DNA and cloned into the pGL3Basic luciferase vector (Promega) (28, 29). A common 3’ primer was used for all the constructs except P1. The primers used were: 5’-AAGCTTGGCCACACAGGCGGCTG-3’ and 5’ primers were 5’-GCTAGCGCGGCGCTAAGCGCCG-3’ for P1-P2, 5’-GCTAGCGGCGCTAAGCGCCG-3’ for T1, 5’-GCTAGCGGCGCTAAGCGCCG-3’ for T2, 5’-GCTAGCGGCGCTAAGCGCCG-3’ for T3, 5’-GCTAGCGGCGCTAAGCGCCG-3’ for T4, and 5’-GCTAGCGGCGCTAAGCGCCG-3’ for T5. Primers for the P1 promoter alone were 5’-GCTAGCGGCGCTAAGCGCCG-3’ and 5’-GCTAGCGGCGCTAAGCGCCG-3’. The identity of all Mdm2 promoter constructs was confirmed by sequencing prior to use. Reporter assays were performed according to the manufacturer’s instructions with the dual luciferase system from Promega. Firefly luciferase activity was normalized to Renilla luciferase activity. -Fold activation was calculated by dividing the normalized luciferase activity by the normalized activity obtained in the presence of the vector and reporter plasmid alone.

**Northern Blot Analysis**—Total RNA was purified from 1 × 10⁶ Ptenloxp/loxp or Pten KO/loxp cells using an RNaseasy kit (Qiagen). Fifteen micrograms of total RNA was loaded onto a 1% agarose gel plate containing 5.4% formaldehyde. RNA was transferred to a Hybond-N+ nitrocellulose membrane (Amersham Biosciences) and probed using standard methods. Quantitation was performed with Image Quant (Molecular Dynamics). A 110-bp cDNA fragment of Mdm2 exon 1 and Mdm2 exon 3–4 was used to make radioactive probes for hybridization. The membrane was probed with a fragment of actin RNA as control.

**RT-PCR**—RNA was isolated using RNasequeous kit (Ambion). Five μg of total RNA was reverse-transcribed using oligo(dt) (Promega) as a primer, and the cDNA product of this reaction was amplified by PCR. The amount of cDNA added to each PCR was normalized using actin cDNA as the standard. Actin amounts were measured by limited PCR using the primer pair 5’-CGGTGCGCTAGGCTGCTGGG-3’ and 5’-GTGGCGCGTCTGAGGCCCA-3’. Two primer sets were used to amplify Mdm2. One set (5’-AGCGCGGCGCCTGTCGATG-3’ and 5’-CTGACTCTCCGCGGTCATG-3’) amplifies only RNAs initiated at the constitutive promoter (exon 1), and the second set (5’-GCGCGCTAGGCGAGCCGACG-3’ and 5’-ACAACAACACACAGGAGA-3’) amplifies RNAs initiated at both the constitutive and p53-responsive promoters (exon 2).
Generation of Tet-inducible Pten Cell Line—Pten$^{loxP/loxP}$ cells were infected with the supernatant from Phoenix cells that had been transfected (as per Gary Nolan’s protocol from his web page) with the pRe-vTet-On vector. The infected Pten$^{loxP/loxP}$ cells were selected with 400 μg/ml G418 for 10 days, and then colonies were allowed to grow out under 200 μg/ml G418 selection. Then Pten$^{loxP/loxP}$ cells were cotransfected with 2 μg of P1-P2 Mdm2 luciferase reporter construct, 0.2 μg of p53, and 0.1–1 μg of PTEN expression vector. Mdm2 transcription was measured 24 h post-transfection. For comparison, luciferase activity from each experiment was normalized to the mock transfection and presented as fold changes. Transfection efficiency was normalized to thymidine kinase-driven Renilla luciferase activity. Three independent tests were performed for each experiment, and the mean ± S.D. was presented here.

PTEN Regulates Mdm2 Expression

PTEN Negatively Regulates Mdm2 mRNA and Protein Levels—Our previous study, together with others, indicates that PTEN negatively controls MDM2 phosphorylation and nuclear translocation by antagonizing the PI 3-kinase/AKT signaling pathway (25–28). We also found that both Mdm2 mRNA and protein levels were increased in Pten-null (Pten$^{loxP/loxP}$, Δ/Δ) cells, when compared with its isogenic WT controls.
To determine the mechanism involved, we pretreated cells with actinomycin D, an inhibitor of transcription, and found that it completely abolished the differences between Pten WT and Pten null cells (Fig. 1, A and B, right panels). This result suggests that PTEN controls not only MDM2 phosphorylation and subcellular localization but also negatively regulates Mdm2 transcription.

PTEN Negatively Regulates Mdm2 Promoter P1 Independent of p53—To further study the cis acting elements required for PTEN-regulated Mdm2 transcription, a genomic DNA fragment consisting of both basal (P1) and p53-responsive (P2) promoter regions of the Mdm2 gene was isolated and ligated to luciferase reporter gene (Fig. 2A, P1-P2). The reporter construct was then cotransfected with a fixed amount of p53 but a variable amount of PTEN into PC3 cells, a human prostate cancer cell line lacking both p53 and PTEN. As shown in Fig. 2B, PTEN negatively regulated p53-induced Mdm2 expression in a dosage-dependent manner.

Mechanistically, PTEN may regulate Mdm2 promoter activity by either interfering with p53 binding to the P2 promoter or through a mechanism independent of p53 binding. To distinguish between these two possibilities, a series of truncation mutations were generated containing different regions of the Mdm2 promoter (see diagram in Fig. 2A) and cotransfected into PC3 cells with PTEN and p53. We found that the first 100 nucleotides in the P1 promoter were absolutely required for PTEN regulation because the reporter construct lacking this fragment failed to respond to the suppression effect of PTEN (Fig. 2C, compare P1-P2 with T1). To further determine whether the activity of PTEN depends on the transcription activation of p53 on the P2 promoter, PTEN was cotransfected with the full-length P1 promoter alone or the truncated promoter lacking the first 100 nucleotides in
the P1 promoter region into primary p53-null embryonic fibroblasts (Fig. 2D). PTEN was able to down-regulate Mdm2 expression to the same level in the absence of p53 and p53-responsible promoter P2 but not with truncated promoter P1 (Fig. 2D). Taken together, these results suggest that PTEN negatively regulates Mdm2 transcription by modulating the P1 promoter independent of the p53 protein or the p53 control element.

PTEN Negatively Regulates Mdm2 through Its Lipid Phosphatase Activity—To determine whether down-regulation of the Mdm2 P1 promoter is because of the lack of PTEN phosphatase activity or because of other structural motifs within PTEN, the Mdm2 reporter constructs, P1-P2 and T1, were cotransfected with either WT Pten or PtenC124S (CS), a catalytically inactive mutant, into PC3 cells. As shown in Fig. 3A, WT PTEN, but not the C124S mutant, could significantly down-regulate P1-P2 promoter, confirming that regulation of Mdm2 expression is dependent on the phosphatase activity of PTEN.

To further test whether PTEN controls Mdm2 transcription through its lipid or protein phosphatase activity, we conducted a similar experiment using the PTEN G129E (GE) mutant. PTEN G129E is a mutation isolated from human cancer that is deficient in lipid phosphatase activity but is functional with synthetic peptide substrates. As shown in Fig. 3A, the PTEN G129E mutant behaved similarly to the C124S as they were both unable to down-regulate Mdm2 expression to the same extent as the WT PTEN. On the other hand, there is no difference between WT and mutant PTEN on the truncated Mdm2 promoter lacking the 100 nucleotides in promoter P1. Cells treated with the PI3K inhibitor LY294002 behaved similarly to cells overexpressing WT PTEN (Fig. 3A). These results further demonstrated the importance of the lipid phosphatase activity of PTEN or the PI 3-kinase pathway in PTEN-controlled Mdm2 expression.
Because PTEN-MDM2-p53 forms a self-reinforced circuit in regulating each other's level and activity, it would be interesting to know whether MDM2 could participate in PTEN-regulated transcription of its own promoter. For this, a p53<sup>−/−</sup> Mdm2<sup>−/−</sup> MEF cell line was used. As demonstrated in Fig. 3B, LY294002 or a dominant negative form of AKT could dramatically inhibit transcription activities of full-length (P1-P2) and P1 promoters to the same extent in the absence of both endogenous p53 and MDM2. Again, deletion of the first 100 nucleotides in the promoter P2 region (T1) significantly abolished the aforementioned effects. Thus, PTEN controls Mdm2 transcription by negatively regulating its P1 promoter. PTEN exerts this function through its lipid phosphatase activity by antagonizing the PI 3-kinase pathway independent of p53 and MDM2.

**The Biological Consequences of PTEN-Regulated Mdm2 Transcription**—In human, P1 is a constitutive promoter independent of p53 and yields a long mRNA (L-HDM2) with exon 2 spliced out. Conversely, transcription from the second promoter P2 depends on p53 binding and gives rise to a short transcript (S-HDM2) lacking exon 1 (see diagram in Fig. 4A). To test whether PTEN regulation of the P1 promoter would lead to alteration of the L-Mdm2/S-Mdm2 ratio, the endogenous Mdm2 transcripts in the presence and absence of PTEN were investigated. RT-PCR and Northern blot analyses demonstrated that PTEN loss dramatically increased the expression of Mdm2 transcripts containing exon 1 (Fig. 4B, Mdm2). A

**Exon 1** but not exon 2. Conversely, reintroducing WT Pten but not mutant Pten lacking lipid phosphatase activity into Pten<sup>Δloxp/Δloxp</sup> cells dramatically suppressed L-Mdm2 expression (Fig. 4C, left). Again, overexpression of PTEN in p53-null fibroblasts led to down-regulated L-Mdm2 mRNA but not S-Mdm2 mRNA (Fig. 4C, right).

To determine whether the effect of PTEN on Mdm2 mRNA expression requires de novo protein synthesis, Pten<sup>Δloxp/Δloxp</sup> and Pten<sup>Δloxp/Δloxp</sup> cells were pretreated with cycloheximide, a protein synthesis inhibitor, and L-Mdm2 levels were measured by RT-PCR analysis and compared with untreated cells. Cycloheximide could completely abolish the negative regulation of PTEN on Mdm2 L-Mdm2 mRNA expression (Fig. 4D), suggesting that PTEN controls transcription of Mdm2 by modulating the production or activity of currently unknown transcription factor(s) (see “Discussion”).

**PTEN Controls Mdm2 Isoform Production**—The Mdm2 locus encodes the p90<sup>MDM2</sup> oncoprotein that binds to and inactivates the p53 tumor suppressor protein. A short p76<sup>MDM2</sup> isoform of Mdm2 has recently been identified that lacks the first 49 amino acids of p90<sup>MDM2</sup> and cannot bind p53. Interestingly, p76<sup>MDM2</sup> can antagonize the p90<sup>MDM2</sup>-mediated degradation of p53 and lead to increased level and activity of p53 (4). Using epitope mapping and site-directed mutagenesis, Saucedo et al. (5) showed that the ratio of the two MDM2 protein products, p90<sup>MDM2</sup> to p76<sup>MDM2</sup>, is determined by the relative abundance of L-Mdm2 mRNA; L-Mdm2 predominantly gives rise to p90<sup>MDM2</sup> whereas p76<sup>MDM2</sup> appears to be the product of translational initiation at codon 50 (AUG) in exon 4. To test whether PTEN will alter the ratio of p90<sup>MDM2</sup> to p76<sup>MDM2</sup>, we measured the endogenous MDM2 protein products in the isogenic Pten<sup>Δloxp/Δloxp</sup> and Pten<sup>Δloxp/Δloxp</sup> cells, using an antibody against both p90<sup>MDM2</sup> and p76<sup>MDM2</sup>. As shown in Fig. 5, PTEN negatively regulates the p90<sup>MDM2</sup> isoform and enhances the ratio differences between p76<sup>MDM2</sup> and p90<sup>MDM2</sup> (Fig. 5, lanes 1 and 2).

To test whether the isoform regulation is directly correlated with PTEN protein levels, an inducible system was generated in which Pten expression can be controlled in a tet-dependent manner in Pten<sup>Δloxp/Δloxp</sup> cells. Re-expression of Pten led to suppression of the p90<sup>MDM2</sup> isoform without significant change in p76<sup>MDM2</sup> (Fig. 5, lanes 3 and 4), resulting in an increased p76<sup>MDM2</sup>/p90<sup>MDM2</sup> ratio as compared with untreated Pten<sup>Δloxp/Δloxp</sup> cells (Fig. 5). Similarly, inhibiting PI 3-kinase activity by LY294002 treatment also increased the p76<sup>MDM2</sup>/p90<sup>MDM2</sup> ratio (Fig. 5, lane 5). This result suggests that PTEN functions by regulating not only the PI 3-kinase pathway or

**Fig. 7. Model of regulation of Mdm2 expression by PTEN.** PTEN can modulate MDM2 functions by two mechanisms, and both depend on its lipid phosphatase activity. By inhibiting PI3K/AKT activity, PTEN negatively regulates phosphorylation of MDM2 and its subsequent nuclear translocation, thus augmenting both the level and function of p53. In addition, PI3K, via a currently unknown transcription factor, down-regulates the Mdm2 P1 promoter, reduces L-Mdm2 production, and effects a change in p76<sup>MDM2</sup>/p90<sup>MDM2</sup> ratio and activities.
p53 protein level and activity but also by regulating Mdm2 expression and isoform production.

PTEN Negatively Regulates L-Mdm2 Expression and p90HDM2 Isoform Production in Vivo—To further support the function of PTEN in regulating Mdm2 transcription, we turned our attention to the Pten-null prostate cancer models generated recently in our laboratory (34) because PTEN loss has been highly associated with both human and murine prostate cancer development. cDNAs were prepared from individual prostate ventral lobes of 9-week-old WT (Ptenlox/lox/PB-Cre5) and Pten homozygous deletion MUT (Ptenlox/lox/PB-Cre^4) male mice. RT-PCR analysis showed complete Pten deletion (Pten Exon 5) and the dramatic increase of Mdm2 exon 1 expression in Pten-deleted prostate tissue (MUT, Fig. 6A), accompanied by an ~10-fold increase in p90HDM2 isoform production, as compared with the WT prostate tissues (Fig. 6B). These results provide strong evidence that PTEN down-regulates Mdm2 in vivo in a manner similar to what we observed in cell lines and suggest that altered Mdm2 level and activity may contribute to tumor formation caused by PTEN loss.

DISCUSSION

In this study, we demonstrated a novel mechanism by which PTEN controls the expression and function of the MDM2 oncoprotein; PTEN, by antagonizing the PI 3-kinase pathway, negatively regulates the P1 promoter on the 5' end of the Mdm2 gene, leading to decreased L-Mdm2 expression and reduced p90HDM2 isoform production. Because the p90HDM2 isoform is capable of binding and inactivating p53, this novel mechanism, together with AKT, controlled MDM2 phosphorylation, and nuclear translocation (25–28) may be critically important for controlling the basal levels as well as the activities of p53 and MDM2 (Fig. 7).

A key issue that remains to be addressed is the identification of the transcription factor(s) that binds to the P1 promoter. Blocking de novo protein synthesis by cycloheximide would be effective enough to even out the differences of L-Mdm2 expression caused by PTEN. This experiment suggests that PTEN regulates Mdm2 exon 1 expression through cooperation with the required newly synthesized protein(s), which are components of transcriptional machinery that have been undergoing continuous turnover.

Because transcriptional regulation of Mdm2 P1 promoter activity by PTEN can be mimicked by blocking the PI 3-kinase activity and depends on de novo protein synthesis, the production or activity of this transcription factor(s) is most likely under the control of the PI 3-kinase pathway. Although transcription factors, e.g. AP-1 and Ets (30), are able to modulate Mdm2 transcription, both of them have been characterized to work through the p53 responsible promoter P2. Our preliminary analysis on DNA sequences corresponding to either P1 or the first 100 nucleotides of the P1 promoter did not identify binding sites of any known transcription factors. Future work is needed to identify such transcription factors and signaling effectors downstream of PI3K responsible for the regulation of the P1 promoter.

Our study suggests that differential promoter usage may contribute to alternative splicing products that lead to preferential MDM2 isoform expression (4, 32). Many previous studies have shown that alternative splicing and internal translational initiation both contribute to the regulation of MDM2 function. Overexpression of HDM2 has been observed in many primary human cancers. Increasing evidence suggests that, besides regulating p53 levels and functions, MDM2 can also participate in tumorigenesis independent of p53 (31, 33). In human prostate carcinoma, overexpression of MDM2 has been frequently observed (35). p21waf1/cip1, which is involved in the development of androgen independent prostate cancer, is down-regulated by MDM2 independently of p53 (20, 36). All these data support our observation that, accompanied with Pten deletion in the murine prostate tumor, Mdm2 mRNA and protein levels are both increased, which may consequently lead to the development of prostate cancer.

In conclusion, in the presence of p53, PTEN up-regulates the p53 level as well as its activity by down-regulating Mdm2 transcription and p53 binding activity. However, in the absence of p53, PTEN may play a role in suppressing MDM2-mediated tumorigenesis through regulation of Mdm2 transcription as well as isoform production. It is important to further investigate the functional linkage between PTEN and p76HDM2 or other MDM2 isoforms that can be modulated by PTEN in human cancer samples, and overexpression of MDM2 may contribute to the tumorigenic process initiated by PTEN mutation.

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