p53 Regulates the Expression of the Tumor Suppressor Gene Maspin

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Zhiquiang Zou‡‡, Chunling Gao†, Akhilesh K. Nagaič‡, Theresa Connell‡, Shin’ichi Saito†, Judd W. Moul‡, Prem Seth*‡‡, Ettore Appella‡§, and Shiv Srivastava‡‡

From the ‡Department of Surgery, Center for Prostate Disease Research, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, the †Department of Surgery, Urology Service, Walter Reed Army Medical Center, Washington, DC 20307, the **Medical Breast Cancer Section, Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, and the ¶Laboratory of Cell Biology, National Institutes of Health, Bethesda, Maryland 20892

Maspin has been shown to inhibit tumor cell invasion and metastasis in breast tumor cells. Maspin expression was detected in normal breast and prostate epithelial cells, whereas tumor cells exhibited reduced or no expression. However, the regulatory mechanism of maspin expression remains unknown. We report here a rapid and robust induction of maspin expression in prostate cancer cells (LNCaP, DU145, and PC3) and breast tumor cells (MCF7) following wild type p53 expression from an adenovirus p53 expression vector (AdWTP53). p53 activates the maspin promoter by binding directly to the p53 consensus-binding site present in the maspin promoter. DNA-damaging agents and cytotoxic drugs induced endogenous maspin expression in cells containing the wild type p53. Maspin expression was refractory to the DNA-damaging agents in cells containing mutant p53. These results, combined with recent studies of the tumor metastasis suppressor gene KAI1 and plasminogen activator inhibitor 1 (PAI1), define a new category of molecular targets of p53 that have the potential to negatively regulate tumor invasion and/or metastasis.

Maspin was originally identified in normal breast epithelial cells (1). The maspin gene encodes a 42-kDa protein and belongs to the serine protease inhibitor (serpin) superfamily with tissue plasminogen activator inhibitor as the possible pro tease target (2). Maspin expression was detected in normal breast and prostate epithelial cells; however, tumor cells showed a decreased expression or absence of expression. Expression of maspin in breast tumor cells inhibit tumor cell invasion in vitro and tumor cell metastasis in vivo (1). Neutralization of maspin by an anti-maspin antibody abolished the invasion suppressive effect of conditioned medium from cultured breast myoepithelial cells on tumor cells (3). A recent report also suggests that the tumor suppressive effects of manganese-containing superoxide dismutase in human breast cancer cells could result from the up-regulation of maspin (4). Gamma linolenic acid, an essential fatty acid with anticancer properties, is reported to induce maspin expression and affect the motility of cancer cells (5). Transcriptional activity of maspin expression differed between prostate normal and tumor cells (6). These observations suggest that maspin expression plays important roles in regulating tumor cell invasion and metastasis. Thus, an understanding of the regulation of maspin expression is important in designing therapeutic agents for the cancer treatment.

Molecular targets of p53, e.g. p53-regulated genes or p53-interacting proteins, have provided critical information central to the current understanding of the biochemical and biologic function of the p53 tumor suppressor gene. The function of p53 as check point protein is now well established (7). p53-regulated genes have also defined the role of p53 in apoptosis, hypoxia, and angiogenesis (8–10). However, the downstream targets of p53 remain to be defined in the process of cancer cell invasion/metastasis. In our search for molecular targets of p53 involved in cell invasion and metastasis, we have now discovered that maspin expression is regulated by wild type (wt)1 p53. In this report, we provide biochemical and cellular biologic evidence demonstrating that maspin is directly regulated by p53.

MATERIALS AND METHODS

Cell Culture—Prostate tumor cell lines DU145, LNCaP, and PC3, breast tumor cell line MCF7, and colon carcinoma cell line HCT116 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in the growth medium recommended by the supplier. Experimental conditions for infection of the cells with recombinant adenovirus vectors expressing wild type p53, p21waf1/cip1, or p27 have been described previously (11, 12).

Northern Blot and Western Blot Analyses—Total cellular RNA was extracted from cells by RNAzol method (Life Technologies, Inc.). Ten µg of total RNA were fractionated on a 1% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a solution (5× SSC, 5× Denhardt’s solution, 40% formamide, 10% dextran sulfate, 10 mM Tris-HCl, pH 7.5) containing a randomly labeled 32P cDNA probes exposed to x-ray film. The DNA fragments used for hybridization were generated by PCR from normal prostate cDNA (CLONTECH). The maspin probe was a 585-bp PCR product spanning nucleotides 576–1160. The p21waf1/cip1 was a 318-bp fragment spanning nucleotides 1745–2035 of the p21waf1/cip1 cDNA. The p27 probe was a 597-bp fragment spanning nucleotides 5–10 of the p27 cDNA. The identity of PCR-derived probes was confirmed by DNA sequencing. A maspin monoclonal antibody was obtained from PharMingen. Total cellular lysate was separated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Western blot analysis was performed using the ECL system.

1 The abbreviations used are: wt, wild type; mt, mutant; pM-Luc, maspin promoter-luciferase reporter; PCR, polymerase chain reaction; bp, base pair(s).
In the gel shift assay.

was mutated to the sequence shown in the mutant oligonucleotide used

297) as the template. The p53 binding site

tagena and using pM-Luc(297mt2) were generated by PCR-based site-directed mu-

ment from pM-Luc(297) was made by deleting the

pM-Luc(1) was made by deleting the

Pst I and

Hin I site) and GAGAACGGCTTCTGCTCCTACC (with the HindIII site).

The pM-Luc(−596) was generated by primers GAGACTGAGGT-

TGTTCTCAACTCTCTG (with the XhoI site) and GAGAAAGCTTA-

GAAGCAGGGTGCTCACC (with the HindIII site). The DNA frag-

ment was cloned into the XhoI and HindIII sites of the pG3 basic vector (Promega). pM-Luc(−297) was made by deleting the PsI fragment

from pM-Luc(−759). The three constructs ended at +87 nucle-

otides from the transcription start site of the maspin. The sequence of the constructs was verified by DNA sequencing. pM-Luc(−297mt1) and pM-Luc(−297mt2) were generated by PCR-based site-directed muta-

genesis using pM-Luc(−297) as the template. The p53 binding site

was mutated to the sequence shown in the mutant oligonucleotide used

in the gel shift assay.

Transfection and Luciferase Assay—The cells were plated at 5 × 10

5 cells/well (6 wells/plate) 1 day before the transfection. The transfection

was performed using the calcium phosphate method (CLONTECH).

The maspin promoter-reporter plasmid (5 μg), the p53 plasmid (2.5 μg),

and an internal control plasmid, pRL-TK (0.5 μg), were cotransfected into cells for 48 h, and the cells were harvested for the luciferase assay.

Luciferase activity was measured by a luminometer using the Dual-

Luciferase reporter assay system (Promega). The results are presented

as the -fold induction of the reporter plasmid alone after normalization

with the internal control plasmid pRL-TK.

Gel Mobility Shift Assay—The gel mobility shift assay was performed as described previously (14). Briefly, labeled oligonucleotide probes (2 ng) were incubated with 30 ng of nonspecific competitor DNA and 50 ng of wt p53 (purified from the baculovirus expression system) in 50 μl

Tris, 100 mM NaCl, 1 mM dithiothreitol at 4 °C for 30 min. The PAb-421

antibody was added after 30 min of incubation with wt p53. The com-

plexes were analyzed on a native 12% polyacrylamide gel. The concen-

tration of the protein was adjusted so that it formed an approximately

50:50 complex with the probes.

RESULTS AND DISCUSSION

To determine whether the tumor suppressor p53 regulates maspin expression in prostate tumor cells, maspin mRNA expression was analyzed in the metastatic prostate cancer line DU145 following wt p53 expression via an adenovirus vector, AdWTP53. DU145 cells were infected with AdWTP53 or the control vector dl312 and were harvested at different time points for RNA isolation. The induction of maspin expression was first noted 3 h after AdWTP53 infection and reached a plateau by 6 h that persisted to at least 48 h (Fig. 1A). The control vector did not increase maspin expression under the same conditions. The kinetics of p53-mediated maspin induc-

tion was rapid and was similar to the kinetics of expression of the well known p53-regulated gene, p21waf1/cip1. To demon-

strate that maspin was specifically induced by p53, DU145 cells infected with adenovirus vectors expressing p21waf1/cip1 or p27 were analyzed for expression of maspin (Fig. 1B). p21waf1/cip1 and p27 were highly expressed but did not induce detectable maspin expression. Only p53 stimulated the maspin expression. These results suggested that maspin expression was specifically induced by p53 and was not the result of nonspecific effects of cell growth arrest and/or apoptosis. p53 induction of maspin expression was also noted in prostate tumor cell lines PC3 and LNCaP and in the breast tumor cell line MCF7 (Fig. 1C).

DNA-damaging agents and cytotoxic drugs are inducers of p53 expression that lead to the induction of downstream target genes, e.g. p21waf1/cip1. To demonstrate whether maspin expression is inducible in response to DNA damage, cells were UV-irradiated or treated with etoposide (VP16). As shown in Fig. 2, UV irradiation or etoposide treatment induced maspin expression in LNCaP cells containing endogenous wt p53; however, DU145 cells harboring mutant p53 did not respond to such treatment. UV irradiation increased maspin expression in

MCF7 cells containing wild type p53 but not in PC3 cells null for p53 protein (data not shown). We repeated this experiment in colon tumor cells (HCT116) that contain wt p53. In addition to UV and etoposide (VP16), maspin expression was also induced by γ irradiation and Adriamycin in HCT116 cells (data not shown).

To demonstrate that p53 directly regulates maspin expression, we conducted gel shift assays to analyze the binding of purified p53 protein to the maspin promoter. Sequence analysis of the maspin promoter revealed three imperfect consensus p53 binding sites in the region between −297 and the transcription start site. As shown in Fig. 3, p53 protein exhibited binding to two of the oligonucleotides (CCGGACATGTGGGAGCCCTT-TGA and TGTTGGACAAAGCTGCCAGGGCTTGAAG) that contain the p53 consensus sequence. The binding was supershifted by adding the p53 antibody, Pab421. When mutations (indicated by underlining) were introduced in the
following oligonucleotides, the p53 protein failed to bind to the oligonucleotides: CCCTATATACAACGAGGCCTTTTGGA, TGTCCTGTAGCTACCTAGACCGTTGAGTAGG. These results provide evidence that p53 has the potential to bind the maspin promoter.

Although we demonstrated that wt p53 activated the transcription of the endogenous maspin gene, to further confirm that p53 directly regulates maspin expression, we used a maspin promoter-luciferase reporter assay to examine whether p53 activates the promoter of the maspin gene. Three constructs with varying lengths of the maspin promoter were used. Lanes 1-3, p21\textsuperscript{wt} (5 μg/ml); lanes 4-6, maspin oligo 1 wt; lanes 7-9, maspin oligo 1 mt; lanes 10-12, maspin oligo 2 wt.; lanes 13-15, maspin oligo 2 mt. The open arrowhead indicates the p53-oligo complexes, and the solid arrowhead indicates the p53-Pab421-oligo complexes.

**FIG. 3.** Binding of p53 to the maspin promoter. The gel mobility shift assay was carried out as described under “Materials and Methods.” A p53 consensus oligonucleotide from the p21\textsuperscript{waf1/cip1} promoter (5'-CCCGAACATGCTCAACATGTTGGGA-3') was used as control. The oligonucleotides from the maspin promoter are: oligo 1, 5'-CCCGAACATGTTGGAGGCCTTTTGGA-3'; oligo 1 mutant, 5'-CCCTATATACAACGAGGCCTTTTGGA-3'; oligo 2, 5'-TGTCCTGTAGCTACCTAGACCGTTGAGTAGG-3'; oligo 2 mutant, 5'-TGTCCTGTAGCTACCTAGACCGTTGAGTAGG-3'. The 32p end-labeled oligonucleotides were incubated with p53 with or without p421, and the complexes were analyzed on 12% native polyacrylamide gels. Lanes 1-3, p21\textsuperscript{wt}; lanes 4-6, maspin oligo 1 wt; lanes 7-9, maspin oligo 1 mt; lanes 10-12, maspin oligo 2 wt.; lanes 13-15, maspin oligo 2 mt. The open arrowhead indicates the p53-oligo complexes, and the solid arrowhead indicates the p53-Pab421-oligo complexes.

**FIG. 4.** Activation of maspin promoter. Top panel, diagram of the maspin promoter-luciferase constructs. The p53 consensus-binding site is indicated. R is a purine, Y is a pyrimidine, and W is adenine or thymidine. Lowercase letters in the maspin binding sites indicate the mismatches. pM-Luc(-297) was used to generate the mutant constructs pM-Luc(-297mt1) and pM-Luc(-297mt2) in which the binding sites were mutated to the same sequence as the mutant oligonucleotide used in the gel shift assay. Maspin promoter reporter (5 μg) plasmid DNA was cotransfected with either pcDNA3p53 wt (2.5 μg) or pcDNA3p53mt (2.5 μg) and an internal control plasmid pHl-TK (0.5 μg) into PC3 cells (5 × 10\textsuperscript{5} cells/well) for 48 h. The cells were lysed in lysis buffer provided by the manufacturer (Promega). The control vector and maspin promoter reporter were cotransfected with the pcDNA3 vector (2.5 μg) for control purposes. Luciferase activity was measured on a luminometer and is reported in arbitrary units. The data are presented as -fold induction over the reporter construct alone. The same experiment was repeated three times in DU145 and LNCaP cells. Bar, S.E.
cloned into a luciferase reporter pGL3-basic vector as described under “Materials and Methods” and as indicated in the top panel of Fig. 4. The promoter region used in these constructs contains the full promoting activity as demonstrated previously (6). The expression of wt p53 or mt p53 (codon 245, Gly → Asp) was driven by a cytomegalovirus promoter (pcDNA3). The maspin promoter-luciferase reporter plasmid was co-transfected with the wt p53 or mt p53 expression vector. The results showed that wt p53 induced luciferase activity of the maspin promoter-reporter by more than 3–4-fold compared with the activity of the reporter plasmid alone (Fig. 4). The mt p53 expression vector was not able to induce luciferase activity under similar conditions. All three constructs showed similar induction by the wt p53. These results suggest that the p53-responsive element must reside within the −297 to +87 region relative to the transcription start site. There are two p53 binding sites in this region. The shortest promoter construct, pM-Luc(−297), was then mutated by site-directed mutagenesis at the p53 binding sites to generate constructs pM-Luc(297mt1) and pM-Luc(−297mt2). The mutant sequences in these constructs were the same as the mutant oligonucleotides used for gel shift assays (Fig. 3). As shown in Fig. 4, with the construct containing a mutant binding site 1, pM-Luc(−297mt1), the activation of the promoter was completely abolished. When binding site 2, pM-Luc(−297mt2), was mutated, the activation of the promoter was not affected. This result suggests that binding site 1 is likely to be the functional site for p53 activation.

In this report we demonstrate a rapid wt p53-dependent induction of maspin expression in prostate and breast tumor cells. These results suggest a role for wt p53 in the regulation of the maspin function involved in cell invasion or metastasis. A recent study has shown that p53 activates the expression of the metastasis suppressor gene KAI1 (15). KAI1 expression was decreased during prostate tumor progression and low expression of KAI1 correlates with the loss of p53 function. Despite the variable frequency of p53 mutations reported in primary prostate tumors, metastatic tumors consistently exhibit a higher incidence of p53 mutations (16, 17). Although there is only one report on the analysis of maspin expression in prostate cancer cells, it is notable that maspin expression was down-regulated in prostate tumor cell lines similar to the findings in mammary tumors cell lines. In an earlier report, Zou et al. (1) have already demonstrated that breast cancer cell-harbor ing maspin expression vectors exhibit decreased cell invasion in vitro and reduced metastasis in vivo. On the basis of the known biologic functions of maspin and this study showing regulation of maspin expression by wt p53, we suggest a functional interaction of p53 and maspin in cell invasion. This report, along with other studies (15, 19), underscores the broader implications of these findings and emphasizes the role of p53 in the negative regulation of cell invasion and metastasis. p53 may suppress tumor metastasis by up-regulating metastasis suppressor genes, e.g. maspin, KAI1, and PAI1. This newly emerging p53 function may provide a mechanistic explanation for the increased metastatic susceptibility of tumors harboring p53 mutations.

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