Identification of functional p53-binding motifs in the mouse wig-1 promoter

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Abstract We previously identified wig-1 as a p53-induced mouse gene that encodes a nuclear zinc finger protein with unknown function. To investigate whether wig-1 is a direct target of p53-dependent transactivation, a DNA fragment corresponding to the promoter region was cloned and sequenced. Three regions containing consensus p53-binding sites were identified. Two p53-binding motifs formed DNA–protein complexes with p53 and were able to drive p53-dependent transcription in a luciferase reporter assay. Our results demonstrate that wig-1 is a direct target of p53-mediated transcriptional transactivation.

Key words: wig-1; p53-Binding site; Promoter

1. Introduction

The tumor suppressor p53 induces cell cycle arrest and/or apoptosis in response to diverse cellular stress conditions such as DNA damage, oncogene activation, hypoxia, and telomere erosion (reviewed in [1,2]). More than half of all human tumors carry mutated p53. This disrupts p53-induced apoptosis, leading to genetic instability and selection for more malignant variants. Tumors that retain wild-type (wt) p53 may frequently carry other defects in the p53 pathway [3]. p53 null mice develop normally in most cases, indicating that p53 is not essential for embryonic development. However, p53 null mice are highly tumor prone and develop lymphomas at an early age [4], underscoring p53: s central role as tumor suppressor.

p53 triggers its biological effects by transcriptional transactivation of specific target genes. This involves binding to a conserved sequence site that consists of two copies of the 10 bp sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0–13 nucleotides [5]. A vast number of p53-regulated genes have been identified. p21 encodes an inhibitor of cyclin-dependent kinases that induces G1 cell cycle arrest. Bax, Noxa, PUMA, and p53AIP1 [6–9] are all involved in cytochrome c release from mitochondria during the initial steps of apoptosis. Genes involved in death receptor-mediated apoptosis, e.g. Fas, KILLER/DR5 and PIDD, are also induced by p53 [10–12]. Nevertheless, no single gene has so far been identified as the main effector of p53-dependent apoptosis. In addition to genes involved in G1 arrest and apoptosis, p53 also induces genes implicated in maintaining genomic integrity such as Reprimo, Gadd45, and 14–3-3δ [13,14] through induction of G2/M arrest.

We have previously identified wig-1 as a gene upregulated in response to activation of wt p53 [15]. wig-1 encodes a zinc finger protein containing three zinc fingers of the Cys_2His_2 type and a nuclear localization signal. We recently cloned and characterized human wig-1. The overall amino acid identity between mouse and human wig-1 proteins is 87%. Human wig-1 was shown to inhibit growth in a colony formation assay [16].

To investigate the mechanism responsible for induction of wig-1 mRNA by wt p53, we studied the interaction between p53 and the mouse wig-1 promoter. Here we report the identification of three regions in the wig-1 promoter that contain p53-binding motifs. Two of these regions were found to bind to p53 in an electrophoresis mobility shift assay (EMSA) and also to confer p53-dependent activation of a reporter plasmid containing the wig-1 promoter. This demonstrates that wig-1 is a bona fide target gene of p53.

2. Materials and methods

2.1. Cloning of the wig-1 promoter

Mouse wig-1 genomic clones were isolated by screening a mouse genomic lambda library (Strain 129Sv/Ola) in λ*Fix*II (provided by B. Vennström, Karolinska Institutet, Sweden), using the wig-1 open reading frame (ORF) as a probe. A lambda clone that contained 5′ sequences of the wig-1 gene was sequenced with the forward primer 5F (5′-ACAATCAGCCTCCAGCAGCGG-3′) and the reverse primer 119R (5′-AACCCGAACCGGCAGGCC-3′), both located within exon 1 of the wig-1 gene. A 1476bp *Pst*I–*Xho*I fragment was cloned into the pBluescriptIIKS vector (pBIIKSwig1promoter).

2.2. EMSA

Recombinant GST-p53 protein was expressed and purified from *Escherichia Coli* as described [17]. The probes (see below) were 5′-end labeled using T4 polynucleotide kinase (Invitrogen, The Netherlands) and [γ-^{32}P]ATP (Amersham, Sweden). After labeling, the probes were purified in a 20% native polyacrylamide gel and eluted in 5 mM $MgCl_2$. EMSA was performed using 50 ng of GST-p53 protein incubated with 0.3 ng probe and 0.3 μg PAb421 monoclonal antibody in binding buffer [20% Glycerol, 25 mM HEPES pH7.4, 50 mM KCl, 1 mg/ml bovine serum albumin, 0.05% Triton-X, 2 mM $MgCl_2$, 10 mM DTT] for 30 min at room temperature. Samples were electrophoresed on a 4% native polyacrylamide gel, fixed in 10% ethanol/10% acetic acid for 10 min, dried and analyzed by autoradiography.

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Abbreviations: BM, binding motif; EMSA, electrophoresis mobility shift assay; MEF, mouse embryonic fibroblast; mt, mutant; ORF, open reading frame; TSS, transcription start site; wt, wild-type

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Probes used for EMSA were BM1 (5′-AGGCCAGCCAGGGCCC-AGCATGGTCCCAGCTTACAAGAGT-3′), BM2 (5′-ATACAAG-TATTCACATGCAGAAGGTACAAGCCGGCAGAACAAGCTA-3′), and BM3 (5′-CCGCTTGTCCGTGCACATGCGC-3′). Mutant (mt) probes used for competition experiments were mtBM1 (5′-AGG-ACATCCAGGGCCCAGAATTGTCCCAGCTAAAAATAGT-3′), mtBM2 (5′-ATAAAATTATTCAAATTCAGAAGGTAAAATCCG-GCAGAAAAATCTA-3′) and mtBM3 (5′-CCGATTTTCCGTGCA-AATTCGC-3′).

2.3. Luciferase reporter constructs

Luciferase reporter constructs were made in pGL3basic (Promega, WI, USA). The promoter fragments were subcloned from pBIIKS-wig1promoter into the *Sma*I–*Xho*I sites in pGL3basic by cleavage at the 5′ end with the restriction enzymes shown in Fig. 3A, blunted with Klenow and subsequently cleaved with *Xho*I. The BM3 deletion mt (pGL3b-BM2ΔBM3) was made with the Transformer™ site-directed mutagenesis kit (Clontech, CA, USA) using the deletion primer 5′GGTCTGCGGGAGCAGAGTAGCGCTGCCGGG-3′.

2.4. Cells and transfections

p53$^{-/-}$ mouse embryonic fibroblasts (MEFs) were grown in IMDM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were transiently transfected using LipofectAMINE2000 reagent (Invitrogen, The Netherlands) according to the manufacturer's instructions. Briefly, 5×10^4 MEFs were plated in 24-well plates and transfected with 1 μg reporter constructs, 0.5 μg pCMV-p53 or pCMV-p53(175) plasmid, 0.1 μg pCMX-β-gal plasmid, and 2 μl LipofectAMINE2000 per well. pCMX-β-gal plasmid served as an internal control to correct differences in transfection efficiency. Protein lysates were prepared 24 h after transfection and assayed for luciferase activity using the Luciferase Reporter Gene Assay High Sensitivity system (Roche Diagnostics Scandinavia, Sweden) according to manufacturer's description. Light emission was detected by an Anthos Lucy3 luminometer.

3. Results

3.1. Cloning of the promoter region of wig-1

A genomic wig-1 clone was obtained by screening a mouse genomic lambda library (mouse strain 129Sv/Ola) using wig-1 cDNA corresponding to the ORF as a probe. A 1.6 kb *Eco*RI–*Bam*HI fragment that hybridized with a probe corresponding to wig-1 exon 1 and a 1.0 kb *Pst*I–*Pst*I fragment that overlapped with the 5′ end of the *Eco*RI–*Bam*HI fragment were cloned and partially sequenced. A *Pst*I–*Xho*I fragment derived from these two fragments was subcloned and sequenced (GenBank accession number AF479745). This 1476 bp fragment contains 1342 bp upstream of the putative exon 1 and 20 bp downstream (Fig. 1). Analysis of the sequence did not reveal any consensus TATA box. However, two initiation regions (INR) that match the consensus sequence 5′-Py Py A(+1) N T/A Py Py-3′ [18] were found. One INR corresponds to the previously observed transcription start site (TSS) of wig-1 [15]. The other INR, located 45 bp further upstream, corresponds the TSS found for the rat wig-1 homologue PAG608 [19].

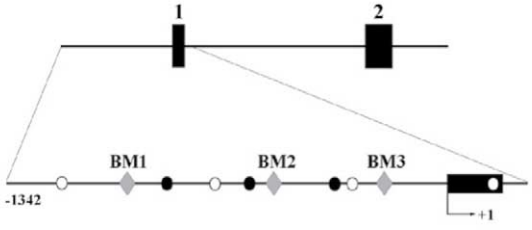
3.2. Identification of p53-binding motifs in the wig-1 promoter

Wt p53 expression results in a rapid induction of wig-1 mRNA, suggesting that wig-1 is a direct transcriptional target of p53. Analysis of the sequence upstream of exon 1 revealed three putative p53-binding motifs, here designated BM1, BM2, and BM3 (Fig. 1). BM1 and BM2 were composed of four decamer motifs corresponding to the consensus sequence for p53 binding. Two decamers overlapped in BM1. BM3 contained two adjacent p53 decamers. All motifs had mismatches compared to the consensus sequence [5]. We also identified three consensus Sp1 binding sites as well as three consensus SOX binding sites in the wig-1 promoter. An additional Sp1 site was located in exon 1 (Fig. 1).

We performed EMSAs to determine if p53 could bind to the putative p53-binding motifs. BM2 and BM3 formed p53 complexes (Fig. 2, lanes 6 and 10) that were efficiently competed out with excess of unlabelled probe (Fig. 2, lanes 7 and 11) but not with an excess of unlabelled mt probe (Fig. 2, lanes 8 and 12). This indicates that p53 interacts specifically with these sites in the wig-1 promoter. BM1 formed a weak complex with p53 (Fig. 2, lane 2) that was efficiently competed out by the mtBM1 probe (Fig. 2, lane 4), indicating that p53 binds unspecifically to this site.

3.3. The wig-1 promoter is p53 responsive

To investigate the functional significance of the p53-binding motifs, we constructed a luciferase reporter driven by the wig-1 promoter (pGL3b-wig1promoter) (Fig. 3A). The reporter was



BM1 -935 AGGCCAGCCAGGGCCCAGCATGGTCCCAGCTAACAAGAGT -914

BM2 -494 ATACAAGTATTCACATGCAGAAGGTACAAGCCGGCAGAACAAGCTA -449

BM3 -160 CCGCTTGTCCGTGCACATGCGC -139

p53 consensus: 5'-RRRCWWGYYY- - 0-13bp - - RRRCWWGYYY-3'

Fig. 1. The promoter region of the mouse wig-1 gene. Exons are indicated as black boxes. Three putative p53-binding motifs were identified by sequence comparison and designated BM1, BM2, and BM3 (shown as diamonds). The sequences of the p53-binding sites are shown with the consensus decamers underlined. Mismatches from the consensus sequence are indicated by stars. The consensus sequence for p53 binding is shown below. R indicates purine, Y pyrimidine, and W adenine or thymine. Putative Sp1 and SOX binding sites in the promoter are indicated as unfilled and filled circles, respectively. Sp1 sites are located at nucleotides −1176 to −1171, −726 to −721, −308 to −303, and +91 to +96 relative to the TSS. SOX sites are located at nucleotides −858 to −850, −602 to −595, and −365 to −358 relative to the TSS.

cotransfected with wt p53 or transcriptionally inactive mt p53(175) [21] expression plasmids into p53 null MEFs. The cells were harvested and assayed for luciferase activity 24 h after transfection. A 12.5-fold induction was observed in cells transfected with wt p53. Mt p53(175) failed to induce wig-1 promoter activity (Fig. 3B), thus indicating that a functional p53 protein is required for transactivation of the wig-1 promoter. The activity observed with mt p53(175) was approximately equal to that observed with empty vector (data not shown).

3.4. Cooperation between p53-binding motifs

To study if all three p53-binding motifs contribute to the activation of the wig-1 promoter, we generated a series of luciferase reporter constructs containing different parts of the wig-1 promoter (Fig. 3A). The reporters were cotransfected and assayed for activity as above. The construct that only contained BM3 (pGL3b-BM3) yielded a 4.6-fold induction with wt p53 as compared to pGL3b-TSS that lacks p53-binding motifs but contains the putative transcription start and exon 1 (Fig. 3B). The construct that also contained the second p53-binding motif (pGL3b-BM2BM3) had an activity close to the maximal activity observed with all three motifs (pGL3b-wig1promoter) (Fig. 3B). Thus, BM1 did not significantly change the total p53-dependent activity of the wig-1 promoter. Therefore, we conclude that BM2 and BM3 are the most important regions for p53-mediated induction of wig-1 promoter activity.

To determine whether BM2 and BM3 act in a synergistic or additive manner, we deleted BM3 in pGL3b-BM2BM3, keeping the surrounding sequences intact (pGL3b-BM2ΔBM3). With this construct we observed a 5.8-fold induction with wt p53, approximately half of that observed for pGL3b-BM2BM3 (Fig. 3B). Thus, it appears that BM2 and BM3 contribute equally to the p53-dependent promoter activity and act in an additive manner.

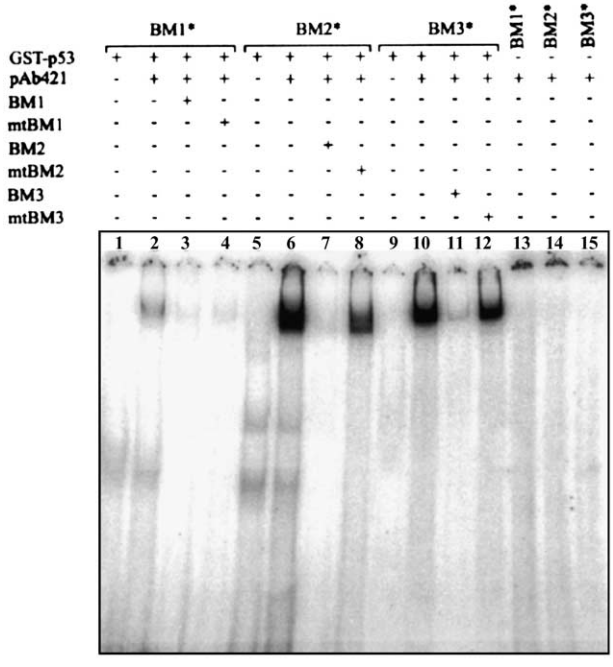


Fig. 2. EMSA showing binding of p53 to the p53-binding motifs in the wig-1 promoter in vitro. Recombinant p53 protein (50 ng) incubated with [α-^{32}P]ATP-labeled oligonucleotides corresponding to the three p53-binding sites. The p53–DNA complexes were resolved on a 4% native polyacrylamide gel. For competition, 10-fold excess of unlabeled oligonucleotides was added to the reactions. The p53 antibody PAb421 was added to activate recombinant p53.

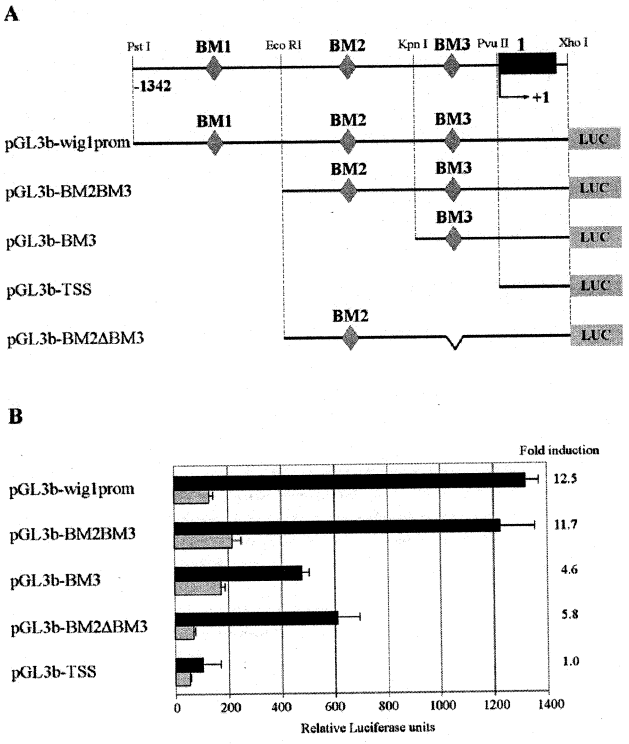


Fig. 3. A: A schematic representation of the luciferase reporter constructs used. Restriction sites used for cloning are indicated. B: p53$^{-/-}$ MEFs were cotransfected with 1 μg of indicated luciferase reporter and 0.5 μg wtp53 (black) or mt p53(175) (gray) plasmid. The data are representative of three independent experiments, each performed in triplicates after normalization with β-galactosidase activity and protein concentrations.

4. Discussion

We have previously shown that wig-1 mRNA is upregulated following p53 activation [15]. This suggested that wig-1 is a direct transcriptional target of p53. To address this question, we have cloned and partially characterized the promoter region of wig-1. Three regions containing between two and four decanucleotide motifs corresponding to the consensus sequence for p53 binding were identified. Two of these, BM2 and BM3, formed DNA–protein complexes with recombinant p53. These results were confirmed by luciferase reporter assays in which both BM2 and BM3 contributed significantly to p53-dependent transactivation. Thus, we conclude that wig-1 is directly transactivated by p53 and is a bona fide p53 target gene.

We observed a weak p53–BM1 complex that was fully competed out by the mtBM1 probe, indicating unspecific binding. Moreover, BM1 had only a minor effect in our reporter assays. The reason for the poor performance of BM1 is unclear. This site contains four p53 consensus decanucleotide motifs, two of which are overlapping. C and G at positions 4 and 7 are conserved in all four motifs (Fig. 1). It is noteworthy, however, that two of the motifs in BM1 have mismatches

within the core sequence C(A/T)(A/T)G of the consensus decamer (C instead of A/T at position 5 of the decamer). In contrast, the motifs in BM2 or BM3 only have mismatches in the stretch of purines or pyrimidines at the beginning or the end of the decamer. Crystal studies of a p53/DNA complex have indicated that the core bases are critical for the interaction between p53 and DNA [20].

The location of p53-binding motifs in target genes varies. In, for example, the p21, MCK, MCG10, PERP and PTEN genes [21–25], one or several p53-binding motifs are located in the promoter region. p53-binding motifs can also be situated in an intron, as in MDM2, Gadd45, and PUMA [8,26,27], or in both the promoter and an intron, like in Cyclin G1 and Bax [28,29]. With regard to the p53-binding motifs, wig-1 shares features with several other p53 target genes. The newly identified MCG10 gene also has three p53-binding motifs in its promoter [23], and like in wig-1 the most distal BM is not functional. Two adjacent p53-binding sites, as in BM2 in the wig-1 promoter, also appear in intron 1 of the MDM2 gene [26]. It should be noted that mouse wig-1 may contain additional unidentified p53-binding sites in introns, as demonstrated for several p53 target genes.

The presence of several p53-binding motifs in the wig-1 promoter may reflect a rather complex transcriptional regulation by p53 and its close relatives p63 and p73, both of which have been shown to bind to the same consensus sequence as p53 and upregulate several of the known p53 target genes (reviewed in [30]). A recent study of the PERP promoter showed that p63 binds both p53-binding sites present in the promoter but more strongly to the 5′ site, whereas p53 was detected only at the 3′ site after DNA damage [31]. It is possible that flanking sequences determine the relative binding affinity of p53, p63, and p73 to the different sites in the promoter, allowing fine-tuned transcriptional regulation in response to various stress-related and developmental signals.

Analysis of the promoter sequence of wig-1 may provide further clues as to the possible involvement of other transcription factors in regulation of wig-1 transcription. p53 uses Sp1 or an Sp1-related protein as a cofactor in order to achieve maximal transactivation of the p21 and Bax promoters [32,33]. Several GC-rich regions in the wig-1 promoter may serve as Sp1 binding sites (Fig. 1). We have also identified three putative binding sites for SOX (SRY-related HMG box) proteins in the wig-1 promoter (Fig. 1). SOX proteins play roles in a number of developmental processes, including spermatogenesis, and several SOX proteins are expressed in testis [34]. It is conceivable that SOX-dependent regulation of transcription is responsible for the observed constitutive expression of wig-1 in adult mouse testis [15]. The functionality of the putative Sp1 and SOX sites in the wig-1 promoter remains to be proven experimentally. In any case, the cloning and characterization of the mouse wig-1 promoter described here should provide a basis for further studies of wig-1 regulation and the biological function of wig-1.

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