Specific recognition of a transcriptional element within the human H-*ras* proto-oncogene by the p53 tumor suppressor

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Abstract. The nuclear phosphoprotein p53 is frequently inactivated in human cancer. Although it was previously classified as an oncoprotein, p53 has emerged as a tumor suppressor controlling cell cycle progression by regulating gene transcription. A major biochemical property of wild-type p53 is its ability to bind DNA in a sequence-specific manner. The human c-H-*ras* gene contains within its first intron sequences that partially match the p53 consensus binding site. We determined that these sequences represent a bona fide p53 element, since *in vitro* translated wild-type p53 recognized them with high affinity. Furthermore, wild-type p53 activated transcription from a reporter plasmid containing the c-H-*ras* element as an enhancer. These findings suggest that p53 regulates cellular growth by coordinate transcription of genes that suppress and promote cellular proliferation.

Introduction

The p53 gene encodes a sequence-specific transcription factor that induces cell cycle arrest or programmed cell death in response to DNA damage (1-6). In more than half of all human tumors p53 is inactivated by missense mutations (7-10). Such tumors become refractory to stimuli that would normally induce apoptosis or cell cycle arrest (11).

The protein encoded by the p53 gene has been studied extensively. The N-terminus of p53 contains a transactivation domain (12,13), the C-terminus a tetramerization domain (14-17), and the central region a sequence-specific DNA binding domain (18-21). The latter domain is inactivated by point mutations in human tumors (7-10).

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The ability of p53 to regulate cell cycle progression and programmed cell death depends on its ability to enhance expression of specific target genes (22). A number of such genes have been identified, including p21/*waf1/cip1* (hereafter referred to as p21), *bax*, *gadd45* and *mdm2* (23-26). The p21 gene encodes an inhibitor of cyclin-dependent kinases (27,28). Its expression leads to arrest of cell cycle progression (6,23). The *bax* gene encodes an antagonist of Bcl-2; Bax promotes, while Bcl-2 inhibits apoptosis (24). The *gadd45* gene is implicated in DNA repair (25). In contrast to p21, *bax* and *gadd45*, all of which suppress cellular proliferation, the *mdm2* gene is a proto-oncogene (29). It encodes a protein that stimulates cell growth by inhibiting the function of two tumor suppressors: p53 and Rb (26,30,31). A common feature of the p21, *bax*, *gadd45* and *mdm2* genes is the presence of p53 binding sites within their regulatory elements (23-26).

The H-*ras* gene, like p53, is frequently mutated in human cancer (32). However, unlike p53, it is a proto-oncogene. It encodes a protein that converts GTP to GDP and acts as a molecular switch in the growth factor signal transduction pathway (33). In human cancer H-*ras* or other members of the *ras* gene family are mutated such that their protein products promote cell proliferation (34-37).

The H-*ras* gene contains within its first intron a sequence that partially matches the consensus p53 binding site (38). We have established that this sequence can function as a p53 DNA binding and transcriptional element raising the possibility that expression of H-*ras* is regulated by the p53 tumor suppressor.

Materials and methods

Recombinant plasmids. Standard cloning procedures were used (39). Plasmid pGEMhp53wtB encodes human wild-type p53 (40). Plasmids pGEMhp53His175B, pGEMhp53Gln248B, pGEMhp53Trp248B and pGEMhp53His273B encode the tumor-derived p53 mutants His175, Gln248, Trp248 and His273, respectively, and were derived from pGEMhp53wtB (40) by site-directed mutagenesis.

Plasmid pSV2hp53wtB was prepared by cloning into the SalI-BglII sites of pSV2humjun (41) a blunted EcoRI-HindIII p53 insert from pGEMhp53wtB (40). Plasmids expressing

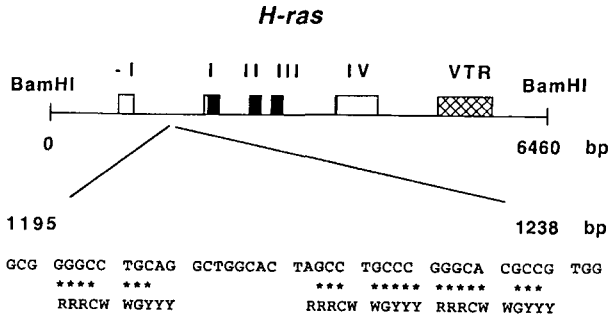

Figure 1. Organization of the human H-*ras* gene and position of the p53 element. The exons are represented by rectangles, the coding sequences as filled rectangles and the VTR by a crosshatched box. The nucleotide sequence of the p53 element is indicated. The p53-specific pentanucleotide repeats are demarcated by dashes and their homology to the p53 consensus site is indicated by asterisks. R, Pu; W, A/T; Y, Py. Nucleotide positions follow Capon *et al* (34).

tumor-derived p53 mutant proteins were similarly derived from the corresponding pGEM plasmids. A pSV2 plasmid without insert was prepared by ligating pSV2humjun (41) linearized with SalI and BglII.

Plasmid pBC12/PLseap contains the coding sequences of secreted alkaline phosphatase with no enhancer or promoter sequences (42). Plasmid pTKseap was derived from pBC12/PLseap and contains a minimal thymidine kinase promoter (43). Plasmids pEp21/TKseap and pEras3HS/TKseap have one copy of oligonucleotides Ep21 and Eras3HS, respectively, cloned in the EcoRV site of pTKseap just upstream of the minimal thymidine kinase promoter. The sequences of oligonucleotides Ep21 and Eras3HS are: CCC-GAACA-TGTCC-CAACA-TGTTG-GGG and GCG-GGGCC-TGCAG-GCTGGCAC-TAGCC-TGCCC-GGGCA-CGCCG-TGG, respectively. The repeats recognized by p53 are underlined.

DNA binding assays. Plasmids of the pGEM series were used to generate *in vitro* translated p53 proteins (21,40,44). These proteins were subsequently incubated with ^{32}P-labeled oligonucleotides and subjected to electrophoresis as previously described (21,40,44). Oligonucleotide TF3 is a non-specific DNA (44). Antibody PAb421 was obtained from Oncogene Science (Uniondale, NY).

Transcription assays. Saos-2 cells were transiently transfected by calcium phosphate precipitation (43). Each transfection was performed using 10 μg of the pSV2 series plasmids driving p53 expression and 20 μg of the reporter plasmids. Alkaline phosphatase activity was determined 48 h later as described (43).

Results

Identification of a putative p53 element in H-ras. DNA sites recognized by the p53 tumor suppressor consist of two half-sites, which may be contiguous or separated by as much as 21 nucleotides (38,40,45). Each half-site is ten nucleotides long and contains two pentamer repeats arranged head-to-head. The sequence of the optimal half-site is GGGCA-TGTCC (38,44). Wild-type p53, however, can also bind to sites that differ from the optimal sequence. It has been suggested that p53 recognizes DNAs containing two half-sites that fit the consensus PuPuPuC(A/T)-(A/T)GPyPyPy, where Pu and Py, are purines and pyrimidines, respectively (45).

A computer search of the genes in the GenBank database has revealed that human H-*ras* contains two consecutive pentanucleotides that fit the p53 consensus (38). We were intrigued by this observation and inspected the adjacent DNA sequences. We were able to identify four more pentanucleotides that together with the ones previously identified, form three putative p53 half-sites (Fig. 1). Two of these half-sites are contiguous, while the third one is located 8 nucleotides upstream. Although none of the half-sites fit the consensus PuPuPuC(A/T)-(A/T)GPyPyPy, their close juxtaposition raises the possibility that they may be recognized by human p53.

Recognition of the putative H-ras element by p53. The ability of human p53 to bind the putative H-*ras* element was examined in an electrophoretic mobility shift assay. *In vitro* translated wild-type p53 bound a ^{32}P-labeled oligonucleotide (Eras3HS) containing all three half-sites of H-*ras* (Fig. 2). Binding was enhanced by antibody PAb421, which recognizes the p53 C-terminus and is known to switch p53 into a conformation with high affinity for DNA (21,40,44, 46,47). Furthermore, PAb421 supershifted the p53/DNA complex, thereby confirming the identity of the DNA binding protein as p53. To determine if recognition of the ^{32}P-labeled H-*ras* DNA by wild-type p53 was sequence-specific, the binding reaction was performed in the presence of 500-fold excess of non-radioactive competitor DNAs. Binding was competed by oligonucleotide Ep21, which contains the p53 element of the p21 gene (23), but not by oligonucleotide TF3, which does not contain a p53 DNA site. Binding was also competed, as expected, by oligonucleotide Eras3HS. In a related experiment we examined binding of p53 to ^{32}P-labeled oligonucleotide Ep21. Wild-type p53 recognized this DNA and furthermore binding was enhanced by antibody PAb421. Excess of non-radioactive Eras3HS DNA competed

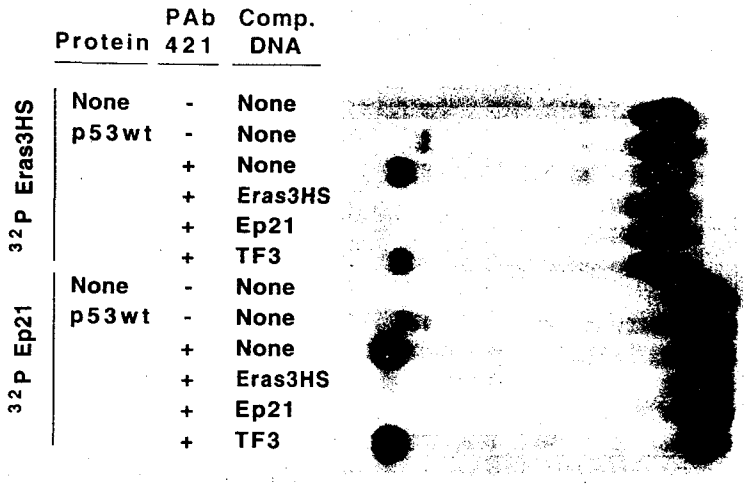

Figure 2. Wild-type p53 recognizes an element of the H-*ras* gene. *In vitro* translated wild-type p53 was incubated with ^{32}P-labeled oligonucleotides Eras3HS or Ep21. Where indicated 500-fold excess unlabeled specific (Ep21) or non-specific (TF3) competitor DNA and/or antibody PAb421 were added. The reactions were resolved on native polyacrylamide gels.

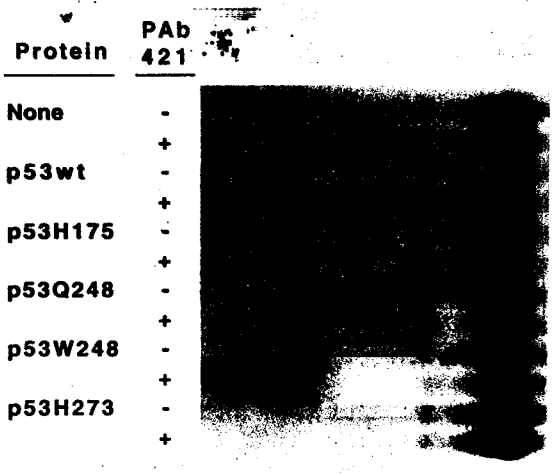

Figure 3. Binding of p53 to the H-*ras* element requires an intact sequence-specific DNA binding domain. *In vitro* translated wild-type p53 or tumor-derived p53 mutants were incubated with ^{32}P-labeled oligonucleotide Eras3HS and then resolved on native polyacrylamide gels. Where indicated the binding reactions were performed in the presence of 0.1 μg antibody PAb421. H, His; Q, Gln; W, Trp.

for binding of p53 to labeled Ep21, while excess of a non-specific DNA (oligonucleotide TF3) did not (Fig. 2). We conclude that p53 recognizes with specificity an element within the human H-*ras* gene. Furthermore, the affinity of p53 for this element is comparable to the well-characterized p53 element of the p21 gene.

Wild-type p53 contains a sequence-specific DNA binding domain within residues 90-290 and a sequence-independent DNA binding domain within its C-terminal 30 amino acids (18-21,48). To determine which domain recognizes the H-*ras* element, we examined tumor-derived p53 mutants, which carry single amino acid substitutions within the sequence-specific DNA binding domain. Mutants His175, Gln248, Trp248 and His273 are very frequently associated with human cancer (7-10). Their names indicate the residue encoded by the mutated codon and the codon number. All these mutants were impaired relative to wild-type p53 in their ability to bind the Eras3HS oligonucleotide (Fig. 3), suggesting that wild-type p53 recognizes the H-*ras* element through its sequence-specific DNA binding domain.

Table I. The H-*ras* p53 element functions as a p53-dependent transcriptional enhancer.

Enhancer/Promoter	Expressed protein	Activity
Eras3HS/TK	Wild-type p53	175.7±3.0
	p53His273	59.7±2.0
	p53Trp248	62.3±13.0
	None	40.0±2.6
Ep21/TK	Wild-type p53	1797.3±420.7
	p53His273	91.3±7.2
	p53Trp248	84.0±3.2
	None	49.7±7.7
None/TK	Wild-type p53	32.0±4.0
None/None	Wild-type p53	0.0±0.0

Wild-type p53 and the tumor-derived p53 mutants His273 and Trp248 were assayed for the ability to activate transcription from reporter plasmids containing Eras3HS or Ep21 enhancer elements. As controls we used a pSV2 expression plasmid without insert (expressing no protein) and reporter plasmids with no enhancer or no promoter. The results are presented as means±1 SE in arbitrary units. TK, thymidine kinase.

H-ras

GGGCC TGCAG GCTGGCAC TAGCC TGCCC GGGCA CGCCG

TGGCGCGCTCCGCCGTGGCC *AGACC TGTTC*

mdm2

GGTCA AGTTG GGACA CGTCC GGCGTCGGCTGTCGGAG *GAGCT* A

AGTCC TGACA TGTCT

p21

GAACA TGTCC CAACA TGTTG

gadd45

GAACA TGTCT AAGCA TGCTG

bax

TCACA AGTTA G AGACA AGCCT

Figure 4. Organization of the H-*ras*, *mdm2*, p21, *gadd45* and *bax* p53 elements. The specific pentanucleotide repeats recognized by p53 are underlined. The H-*ras* and *mdm2* genes contain a fourth p53 half-site (in italics), whose functional significance has not been investigated yet.

The H-ras p53 element functions as a transcriptional enhancer. Wild-type p53 is a known transcriptional activator (12,13). Since the H-*ras* element is specifically recognized by p53, we examined whether it could function as a p53-dependent transcriptional enhancer. Reporter plasmids were constructed that contained the H-*ras* or the p21 or no p53 element upstream of a minimal promoter driving expression of secreted alkaline phosphatase. These reporters together with plasmids directing expression of p53 were transiently transfected into Saos-2 osteosarcoma cells, which lack endogenous p53 (49). Wild-type p53 activated transcription from the reporter plasmids containing H-*ras* or p21 elements, but not from reporters lacking a p53 element or lacking a promoter (Table I). Consistent with their compromised DNA binding activities, the tumor-derived p53 mutants His273 and Trp248 failed to activate transcription from any of the reporter plasmids tested. We conclude that the H-*ras* element, although not as potent as the p21 element, can serve as a p53-dependent transcriptional enhancer.

Discussion

The p53 tumor suppressor controls cell cycle progression by regulating gene expression (22). The genes whose expression is induced by p53 invariably contain p53 binding sites within their regulatory regions (23-26). Interestingly, H-*ras* contains within its first intron sequences with homology to the p53 consensus recognition site (38). We demonstrated in this study that wild-type p53 recognized these sequences with specificity and with affinity comparable to its affinity for the bona fide p21 element. Furthermore, the H-*ras* element functioned as a p53-dependent transcriptional enhancer in the context of an artificial reporter plasmid, albeit with a lower efficiency than the p21 element. We have therefore established the presence of a p53-dependent transcriptional element within the H-*ras* gene. The most likely interpretation of our results is that p53 is a physiological regulator of H-*ras* expression.

Activation of H-*ras* expression by p53 may seem to be a paradox, since p53 is a tumor suppressor (50,51) and H-*ras* a proto-oncogene (32-37). However, precedence has already been established for activation of proto-oncogenes by p53. Wild-type p53 induces expression of *mdm2*, whose protein product functions as an oncoprotein by inhibiting the tumor suppressing activities of both p53 and Rb (26,29-31). Amplification of the *mdm2* gene or enhanced translation are frequently associated with human tumors (52,53). Wild-type p53 may therefore induce expression of two proto-oncogenes frequently associated with human cancer: *mdm2* and H-*ras*.

The list of genes known to be regulated by p53 includes p21, *gadd45*, *bax*, *mdm2* and H-*ras* (23-26). The p21 gene encodes an inhibitor of cyclin-dependent kinases (27,28). Its expression arrests cellular growth (6,23). The *gadd45* gene participates in the cellular response to DNA damage (25), while *bax* stimulates programmed cell death (24). None of these three genes stimulate cell proliferation. In contrast, *mdm2* and H-*ras* are proto-oncogenes. Interestingly, there are certain similarities in the organization of the half-sites in the p53 elements of the H-*ras* and *mdm2* genes, which are not shared with the p53 elements of the other genes targeted by p53. In H-*ras* there are three half-sites (Fig. 4). Two of them are contiguous, while the third one is 8 nucleotides upstream of the first two. In *mdm2* there are again three half-sites (26). Two are contiguous, while the third one is 28 nucleotides downstream. In contrast to H-*ras* and *mdm2*, the half-sites in the p21, *gadd45* and *bax* elements are contiguous (23-25). The organization of the half-sites affects the ability of p53 to recognize these elements. Wild-type p53 reversibly switches between two conformations (21,40,44,46,47). In the 'inactive' T state wild-type p53 adopts dihedral symmetry,

cn that it cannot recognize contiguous half-sites. It can recognize, however, non-contiguous half-sites (40). In the 'activated' R state wild-type p53 is not locked in a dihedrally symmetric state and its DNA binding domains can recognize even contiguous half-sites (40). Thus, the presence of non-contiguous p53 half-sites in the H-*ras* and *mdm2* genes may allow their transcription to be regulated by p53 even when it is in the 'inactive' T state.

The p53 element in H-*ras* is contained within the first intron. The significance of this is not understood at this time. Interestingly, the p53 element of *mdm2* is also within the first intron (26). Transcription of *mdm2* is initiated either at a promoter upstream of the first exon or at a promoter within the first intron. Wild-type p53 activates transcription only from the second promoter (54). Transcripts initiating at both promoters contain the entire *mdm2* coding sequence, but differ in the efficiency with which translation is initiated at codon 1. Thus, the transcripts that include the first exon express mostly an N-terminally truncated Mdm2 protein, while the transcripts whose expression is induced by p53 express full-length Mdm2 (55). The two forms of Mdm2 differ in their functional properties. The full-length form, but not the truncated, can associate with the transcription activation domain of p53 closing a negative feedback loop, whereby p53 activates *mdm2* transcription and Mdm2 suppresses the transcriptional activity of p53 (26). It remains to be determined whether p53 induces expression of transcripts initiating at the first intron of H-*ras*, and whether any functional significance can be ascribed to such transcripts.

In conclusion these studies demonstrate the presence of a p53 transcriptional element in the H-*ras* gene. The p53 tumor suppressor may therefore exert its cellular effects by coordinate activation of genes that suppress and induce cell proliferation.

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