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).

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...
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 Sall and BgIII.

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 32P-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 32P-labeled oligonucleotide 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 32P-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 32P-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 32P-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 32P-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, Gin; 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.
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 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±SE in arbitrary units. TK, thymidine kinase.

Table 1. The H-ras p53 element functions as a p53-dependent transcriptional enhancer.

<table>
<thead>
<tr>
<th>Enhancer/Promoter</th>
<th>Expressed protein</th>
<th>Activity</th>
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<tbody>
<tr>
<td>Eras3HS/TK</td>
<td>Wild-type p53</td>
<td>175.7±3.0</td>
</tr>
<tr>
<td></td>
<td>p53His273</td>
<td>59.7±2.0</td>
</tr>
<tr>
<td></td>
<td>p53Trp248</td>
<td>62.3±13.0</td>
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<tr>
<td></td>
<td>None</td>
<td>40.0±2.6</td>
</tr>
<tr>
<td>Ep21/TK</td>
<td>Wild-type p53</td>
<td>1797.3±420.7</td>
</tr>
<tr>
<td></td>
<td>p53His273</td>
<td>91.3±7.2</td>
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<tr>
<td></td>
<td>p53Trp248</td>
<td>84.0±3.2</td>
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<td>49.7±7.7</td>
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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. We have therefore established the presence of a p53-dependent transcriptional enhancer element within the H-ras gene. The most likely interpretation of our results is that p53 is a physiological regulator of H-ras expression.

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

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.
en 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 elément 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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