A Novel Mechanism for p53 to Regulate Its Target Gene ECK in Signaling Apoptosis

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Abstract

Transcription factor p53 regulates its target genes through binding to DNA consensus sequence and activating the promoters of its downstream genes. The conventional p53 consensus binding sequence was defined as two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ with a spacer of 0 to 13 bp, which exists in the regulatory regions of some p53 target genes. However, there is no such p53 consensus sequence in the promoters of a number of p53-responsive genes, suggesting that there might be other mechanisms whereby p53 transactivates the promoters of its target genes. We report here that p53 uses a novel binding mechanism to regulate the transcription of epithelial cell kinase (ECK), a receptor protein-tyrosine kinase implicated in signal transduction. We show that p53 binds to a 10-bp perfect palindromic decanucleotide (GTGACGTCAC) in the ECK promoter, activates the ECK promoter, and increases the transcription of ECK. This palindrome is required for p53-mediated transactivation of the ECK promoter. ECK is highly responsive to oxidative damage that leads to cell death. Ectopic expression of ECK causes spontaneous apoptosis in breast cancer cells. We found that ectopic expression of a mutant ECK fails to induce apoptosis in cancer cells. Our findings show that p53 is a transcriptional regulator of ECK in mediating apoptosis. The discovery of the novel p53-binding motif in the promoter may lead to the identification of a new class of p53 target genes.

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Introduction

Tumor suppressor p53 plays a fundamental role in cell cycle checkpoint control, cell death induction, and genetic stability (1, 2). As a sequence-specific DNA-binding protein, p53 activates transcription of its target genes through binding to specific DNA consensus sequences. The conventional p53 consensus DNA sequence is defined as two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by 0 to 14 bp (3), which exists in the regulatory regions of some p53 target genes. However, it is often the case where there is no such p53 consensus sequence in many other p53-regulated genes, suggesting that there might be other mechanisms whereby p53 regulates its target genes. Here, we present an example that p53 regulates the transcriptional expression of the receptor tyrosine kinase epithelial cell kinase (ECK) through its binding to a palindromic DNA sequence in the ECK promoter. This palindromic sequence is completely different from the known p53 consensus site (3). Our data further show that ECK is a transcriptional target of p53 in signaling apoptosis.

ECK or EphA2, is a transmembrane tyrosine kinase structurally related to the EPH subfamily (4). ECK protein has an external domain with an NH2-terminal signal peptide, a transmembrane domain, and a cytoplasmic domain, which includes a canonical protein-tyrosine kinase catalytic site in the intracellular region of the protein. ECK is widely expressed in a variety of human tissues. ECK is also expressed predominantly in cell lines of epithelial origin (4). The expression of murine ECK is highly restricted during early development. ECK exhibits a dynamic and spatially restricted expression pattern in the prospective hindbrain region during embryogenesis, suggesting that murine ECK may play a role in guiding early hindbrain development (5). The human ECK maps to 1p36.1, which is a region that is frequently deleted in neuroblastoma, melanoma, and other types of human cancers (6). Studies of ECK in cancer have concentrated primarily on the levels of ECK in clinical samples and cell models. It was reported that EphA2 is regulated by p53, and it induces apoptosis (7). However, there is no consensus binding site in the promoter of the human ECK gene, opening up a question of how this gene is regulated by p53.

Using both PCR-select cDNA subtraction and DNA microarray techniques, we observed that ECK is up-regulated by p53 (8). We further found that p53 regulates the expression of ECK at the transcriptional level. ECK is greatly up-regulated upon the activation of p53 and is increased following oxidative stress. We have identified a potential regulatory region within a 730-bp DNA sequence from the transcriptional initiation site of human ECK gene and found that p53 greatly induces luciferase activity of the luciferase reporter containing this sequence. Furthermore, we show that p53 physically binds to a 10-bp perfect palindromic DNA sequence in the ECK promoter. This palindromic site is completely different from the known p53 consensus site (3). Overexpression of ECK causes spontaneous
apoptosis. Our findings show that p53 is a transcriptional regulator of ECK. Characterization of ECK as a cell death mediator may lead to the development of effective new chemotherapeutic strategies in cancer treatment.

**Results**

Transcriptional Regulation of ECK by p53 in Signaling Apoptosis

p53 has two fundamental functions: cell cycle checkpoint control and cell death induction in response to DNA damage and other genotoxic stresses. It is known that p53 mediates G1 arrest through transactivation of p21. The mechanism whereby p53 mediates apoptosis is one of the hottest areas of current research. To explore the process of p53-mediated apoptosis, we set out to identify new p53 target genes that are involved in the apoptotic process. We previously developed a p53-inducible system in which a p53ER fusion protein, human p53 protein fused to the hormone-binding domain of the human estrogen receptor, is activated by estradiol binding to p53ER fusion protein (9). This p53-inducible system, expressed in H1299, a p53-deficient human cancer cell line, named Hp53ER, allowed us to show the importance of p53 as a transcription regulator in apoptosis (9). The activation of p53 leads to rapid cell death by apoptosis under oxidative stress. We used PCR-select cDNA subtraction to identify genes that are induced by p53 in this inducible system. Two cDNA libraries were made from cells with or without p53 function for subtraction using a PCR-select cDNA subtraction system (Clontech, Palo Alto, CA), according to the manufacturer’s protocol. Briefly, cDNA from the induced cells (Hp53ER) was ligated with two adaptors to be amplified by PCR after subtraction and then hybridized with cDNA from the control cells (H1299) to subtract common cDNA. Differentially expressed transcripts were enriched through subtractive hybridization of the two groups followed by PCR amplification using the two adaptor primers. PCR products were ligated into a T/A cloning vector (Clontech) for sequencing and database analysis. The potentially positive cDNA clones were then tested for their expression in the presence and absence of p53 by reverse transcription-PCR and Northern blot analysis. One of the identified clones contains a sequence identical to the gene for human ECK. We also found that ECK was induced by p53 using a DNA microarray in the EB-1 cell line, named Hp53ER, which induced apoptosis by p53 in this inducible system (8).

To confirm these observations, we used the human ECK coding sequence as a probe for Northern analysis of ECK expression in these two p53-inducible systems. As shown in Fig. 1A, there is a low level of ECK transcript in p53-null H1299 cells (lane 1). The expression of ECK is greatly increased in p53ER cells in the presence of 17β-estradiol that activates the transactivity of p53 (lane 2), suggesting that ECK is induced by p53 at the transcriptional level. To extend our observation of positive regulation of ECK by p53, we used the EB-1 cell line from EB cells derived from a human colon cancer. EB-1 cells contain a stable-transfected wild-type p53 transgene under the control of the metallothionein promoter (10) and express wild-type p53 upon administration of zinc chloride (ZnCl2). These cells undergo apoptosis following serum starvation in the presence of p53 (10). As shown in Fig. 1B, ECK mRNA is increased in EB-1 cells in the presence of 100 μmol/L ZnCl2 only (lane 1 versus lane 3). Significantly, ECK expression is increased even more in the EB-1 cells treated with both 100 μmol/L ZnCl2 and serum starvation (lane 2), which induced apoptosis (10). These results suggest that ECK is up-regulated by p53, and induction of ECK is enhanced when cells undergo apoptosis. To determine whether ECK expression is responsive to genotoxic damage, we treated embryonic fibroblasts derived from mice with differing p53 status with γ-irradiation or H2O2. As shown in Fig. 1C, ECK protein is not induced by γ-irradiation (lanes 1 versus
Transcriptional Regulation of ECK by p53

Transactivation of the Promoter of the Human ECK Gene by p53

To understand the molecular basis for regulation of ECK by p53, we attempted to clone the regulatory region of the human ECK gene. We used a 600-bp fragment flanking the 5'-untranslated region and partial coding sequence of the ECK gene as a probe to screen a human genomic library in the pWE15 cosmid (Clontech) for the promoter of the ECK gene. The screening procedure was done according to the manufacturer’s protocol. The resulting positive clones were subjected to secondary screening. The inserts of five positive clones were digested and subcloned into a pT-Adv vector (Clontech) for sequencing to define the promoter sequence. We obtained two identical clones that contain a 2.3-kb fragment of sequence upstream of the translation initiation site of the ECK gene. As shown in Fig. 2A, there are two 10-bp sequences from −810 to −801 (5'-GGGCATGTTg-3') and −711 to −702 (5'-GtGCATGTCT-3') relative to the translation start site, both of which match (9 in 10) with a known p53 consensus binding site of 5'-PuPuPuPuC(A/T)(T/ A)GPyPyPy-3' (3). Interestingly, there is a 10-bp perfect palindromic decanucleotide at −189 to −180 (5'-GTGACGT-CAC-3') in the ECK promoter, as indicated in bold and underlined letters.

To determine whether the ECK promoter is regulated by p53, three regions of the ECK promoter were amplified by PCR to make luciferase reporters as the following: (a) a 0.7-kb (−882/−153) sequence containing both the imperfect p53 consensus binding sites and the 10-bp palindromic decanucleotide; (b) a 0.3-kb (−882/−524) sequence with two imperfect p53 consensus sites but without the palindromic motif; (c) a 0.4-kb (−553/−153) sequence with the palindromic decanucleotide but without the imperfect p53 consensus sites. As shown in Fig. 2B, the luciferase reporters were constructed by ligating respective sequences into a luciferase reporter vector pGL3-basic (Promega, Madison, WI) adjacent to a luciferase reporter gene, resulting in pGL3/ECK-0.7, pGL3/ECK-0.3, and pGL3/ECK-0.4, respectively. To examine luciferase activity, the luciferase reporters were transfected into H1299 cells along with a pCMV vector as a control, or with pCMV/wild-type p53 expression vector (pCMV/wtp53; ref. 14). As shown in Fig. 2C, there is a basal activity of the ECK promoter in the absence of p53. The activity of the pGL3/ECK-0.7 is greatly induced by wild-type p53 but not by a mutant p53 plasmid (pCM3-248) containing a point mutation at codon 248. The fold induction of the ECK promoter activity is similar to that of the p21 promoter activity by wild-type p53 but much higher than that of either MDM2 or Bax promoter activity, suggesting that the ECK promoter is highly responsive to p53 function. However, there is no induction of promoter activity from the pGL3/ECK-0.3 reporter, which contains two imperfect p53 consensus sites. Interestingly, the luciferase activity is retained by the pGL3/ECK-0.4 reporter, which contains the palindromic site but does not contain the imperfect p53 consensus sites. To confirm the requirement of this palindromic motif for p53 binding, we did a PCR-based site-directed mutagenesis to create a mutant form of pGL3/ECK-0.7, pGL3/ECK-0.7m, which contains a T-to-G mutation in the palindromic site (GTGACGgCAC). Dramatically, the induction of luciferase activity of this mutant reporter by p53 was reduced to the basal level (Fig. 2C). These results show that the palindromic site is crucial for induction of the ECK promoter activity by p53.

Identification of a Palindromic Motif in the ECK Promoter as a Novel Recognition Site for p53 Binding

To determine whether p53 physically binds to this palindromic site, we synthesized a 36-bp oligonucleotide containing the palindromic sequence (ECK oligo 36W) and used it as a probe to perform an electrophoretic mobility shift assay. We also used an oligo containing the conventional p53-binding sequence from the p21 promoter (p21 oligo 30W) as control. As shown in Fig. 3, one prominent shifted band was detected when this 36-bp radiolabeled element was incubated with recombinant human wild-type p53 (lane 2). This gel shift was reduced in the presence of an excess of the unlabeled 36W oligonucleotide (lane 3) but remain unchanged in the presence of nonspecific oligo NS30W (lane 4). To confirm binding specificity, we included an anti-p53 monoclonal antibody in the reaction. The addition of the p53 antibody resulted in a clear supershift band (lane 5). As control, incubation of this labeled p21 oligo 30W probe with p53 protein also resulted in a shifted band (lane 8), which was supershifted by the anti-p53 monoclonal antibody (lane 9). Note that the patterns of gel shift and supershift from both the palindrome and conventional sequence are almost identical. These results show that p53 binds to the element containing the palindromic sequence.

Jin and Yin, unpublished data.
To better define the binding sequence, a series of oligonucleotides containing either the complete or incomplete palindromic sequence with flanking sequences were used for electrophoretic mobility shift assay. As shown in Fig. 4, a specific shift band formed in all reactions containing p53 protein and various probes with the complete 10-bp palindromic sequence. However, there was no shift band generated from the reactions of p53 with incomplete palindromic probes omitting...
even one nucleotide on either side of the palindromic sequence (Fig. 4, oligo 9 and oligo 11). These results clearly show that the perfect 10-bp palindromic sequence is essential for p53 binding. To determine the specificity of each nucleotide in this motif, we generated mutations of the 14-mer oligo sequence containing the palindromic motif with two nucleotides flanking both sides. As expected, p53 binds to the palindromic site, but double mutations of the two flanking nucleotides have no effect on its binding to the palindromic site (Fig. 5, lane 1 versus lanes 2 and 13). However, the change of a single nucleotide in the palindromic site resulted in either partial or complete loss of p53-shifted bands (Fig. 5, lane 1 versus lanes 3-12). Interestingly, mutations of the first nucleotide on either side of the palindromic motif only reduced the affinity of p53 for the palindromic motif, and these mutated oligos still formed weak shift bands with p53 (lanes 3, 10, and 12). These results indicate that whereas the intact palindromic motif is preferred for p53 binding, p53 may interact with the variants of the palindromic motif. It is conceivable that p53 can use this novel binding mechanism to regulate a new class of target genes, which may differ from the genes regulated through the conventional mechanism. In fact, we have searched the human genome database for other genes with this palindromic sequence and found that there are a number of genes containing this identical motif or similar motifs in their promoters. The partial results of genome-wide search are shown in Table 1. Some of these genes are stress inducible and are likely targets of p53 through palindromic sites.

**Mediation of the Cellular Response by ECK to Oxidative Damage and Apoptosis**

The fact that ECK is up-regulated by p53 under apoptotic conditions indicates that ECK may play a role in signaling cell death. To determine the role of ECK in the apoptotic process, we constructed a human ECK expression vector by cloning a 2.9-kb human ECK cDNA containing the full coding sequence in frame into a mammalian expression vector driven by a human cytomegalovirus (pcDNA3/hygro, Invitrogen, Carlsbad, CA). The resulting ECK expression vector (pcDNA3/hECK) or an empty vector pcDNA3 was transfected into MDA-MB435 and MCF-7 cells, which were then selected with hygromycin B for isolating stable clones of MB435/ECK (Fig. 6A, lanes 2 and 3) and MCF7/ECK expressing ECK (Fig. 6A, lanes 7 and 8). We observed that MB435/ECK-10 is highly susceptible to cell killing by H2O2 treatment (Fig. 6B). Interestingly, during culture of these cells, we observed that ~25% of MCF-7/ECK-1 and MCF-7/ECK-7 cells were susceptible to spontaneous cell death. The nature of the cell death is apoptosis, which was determined by terminal deoxynucleotidyl transferase–mediated nick-end labeling assay (Fig. 6C). These results indicate that ECK may function directly as a cell death mediator. Because ECK is a downstream effector in the p53 pathway, it is important to look at the status of ECK sequence during tumorigenesis. We analyzed some human cancer cell lines by sequencing the coding sequence of ECK and found that there is an A:G nucleotide transition (TAC to TGC, tyrosine to cysteine) at codon 87 in exon 3 of ECK in colon cancer cell line DLD-1 and leukemia cell line HL60 (data not shown). To determine whether this mutation influences ECK function, we used PCR-directed site mutagenesis technique to generate a point mutation at codon 87 (TAC to TGC, tyrosine to cysteine) in the pcDNA3/hECK, resulting in the vector pcDNA3/ECK-mt. This vector was introduced into MB435 and MCF-7 cells, respectively, and stable cells expressing mutant ECK were determined by Northern analysis (Fig. 6A, lanes 4, 5, and 9). As shown in Fig. 6B, MB435 cells expressing the mutant ECK (MB435/ECK-mt-7) are highly resistant to cell killing by oxidative damage. In addition, unlike wild-type ECK, overexpression of the mutated ECK in MCF-7 (MCF7/ECK-mt-1) fails to induce spontaneous apoptosis (Fig. 6C). These data suggest that mutant ECK loses normal function of ECK in signaling apoptosis. Therefore, it is possible that mutant ECK in some cancer cells actually possesses oncogenic functions.

* Wang and Yin, unpublished data.

**Fig. 3.** Gel shift analysis of interaction between p53 protein and oligonucleotides containing a palindromic sequence. Purified recombinant human wild-type p53 was incubated with 32P-labeled double-stranded oligonucleotides containing the palindromic sequence GTGACGTCAC present in the ECK promoter or the consensus sequence in the p21 promoter. For competition, 50-fold excess of the unlabeled oligonucleotide was included in reactions. Supershift was carried out by adding p53 monoclonal antibody (PAb 421) in reactions. The protein-DNA complexes were separated by 4% native polyacrylamide gel electrophoresis and visualized by autoradiography. The protein-DNA complexes and free probes are indicated. Sequences of the oligos were listed.
Discussion

We have shown a palindromic motif as a novel binding site for p53 to regulate its target gene ECK during apoptosis. We propose that p53 can use this novel binding mechanism to regulate a new class of target genes. p53 may selectively regulate different groups of their target genes through this mechanism or through the conventional mechanisms. In fact, we have searched the human genome database with this palindromic sequence and found that there are a number of genes containing this site in their regulatory regions. Therefore, the identification of the new mechanism for p53 action will provide insights into the molecular basis of how these tumor suppressors share their targets and cooperate to mediate apoptosis of cancer cells. A well-characterized p53 consensus binding site is two copies of the 10-bp motif 5'-PuPuPuC(A/T) (T/A)GPyPyPy-3', separated by 0 to 13 bp spacer (3). This consensus site has been found in the regulatory regions of a number of genes regulated by p53, such as p21, Bax, Puma, and PIG3 (15-18). However, there are cases where no known p53-responsive consensus sequence can be identified in p53-inducible genes. This is the case in our study of the regulation of ECK. There is only one imperfect p53 consensus site

![Figure 4](image1.png)

**FIGURE 4.** Definition of a palindrome motif as a p53-binding sequence. The double-stranded 32P-labeled oligonucleotides containing either the perfect 10-bp palindromic sequence or the various deletions were incubated with recombinant wild-type p53 as indicated. The shifted bands represent the p53-DNA complex. All oligonucleotide sequences are listed as one strand (5'→3') and numbered with respect to the lanes.

![Figure 5](image2.png)

**FIGURE 5.** Mutational analysis of a new recognition site for p53 binding. Single mutations of a 14-mer sequence containing the 10-bp palindromic site were generated and synthesized as listed. The wild-type and mutated oligos were labeled by 32P-ATP, and the probes were incubated with recombinant human wild-type p53 for EMSA. The complexes of p53 and oligonucleotides were revealed as shift bands as indicated. The oligonucleotides used in each lane are listed and numbered correspondingly.
in the ECK promoter. However, there is no evidence for interaction between p53 and this putative binding site in our experimental assays. Our studies reveal a novel mechanism for transcriptional regulation of ECK by p53. Functional and mutational analyses of the ECK promoter enabled us to elucidate how p53 regulates the ECK promoter. We identified a 10-bp perfect palindromic sequence as a binding site for p53 to transactivate ECK. This is a new line of evidence that p53 uses a single palindromic site in a promoter to regulate its target gene.

Systematic analysis of p53 target genes using DNA microarray technology has shown that p53 can regulate >60 genes with defined functions in many systems with a variety of biological effects (8). The remaining challenge is to elucidate how p53 regulates so many genes and what is the molecular basis for p53 function in response to various factors and environmental stresses. Therefore, the identification of the new palindromic sequence as a second type of p53-binding site will pave the way for better understanding of how p53 plays multiple roles and how other factors influence the selection for p53 binding to a site in its target genes. We have generated sufficient data to classify ECK as a member of the p53-regulated gene family through a novel mechanism. This is consistent with a report that EphA2 (ECK) is regulated by p53 family proteins (7). We observed that ECK is significantly up-regulated by p53 in p53-inducible systems under the conditions of serum starvation and oxidative damage that causes apoptosis. This is an example that p53 directly regulates expression of a receptor tyrosine kinase in response to oxidative damage, leading to cell death.

Our data have established a molecular basis for how p53 regulates uses a new mechanism to regulate its targets. We have recently identified a 10-bp palindromic sequence in the promoter of cytochrome c, which is identical to the palindromic sequence in the ECK promoter. Cytochrome c is located in the mitochondrial membrane, and it plays an essential role in the activation of caspase-related protease cascade during apoptosis (19). We have also found the same palindromic site in the promoter of the human CL100 or mitogen-activated protein kinase phosphatase-1 (MKP-1), which is highly responsive to oxidative stress (20). MKP-1/CL100 is a dual threonine/tyrosine phosphatase that specifically dephosphorylates and inactivates mitogen-activated protein kinases (21). We observed that p53 physically binds to this palindromic site and activates the promoter of the human MKP-1 gene. Furthermore, through genome-wide search, we have found a number of human genes that contain this palindromic site in the regulatory region of these genes (Table 1). We have previously reported a mechanism whereby p53 binds to another form of palindrome to regulates PAC1, a dual specificity protein phosphatases and an inhibitor of mitogen-activated protein kinases (22). Taken together, these findings suggest that p53 may regulate more target genes through binding different palindromes.

If ECK is a critical effector in the p53 pathway, sufficient expression of ECK might result in the same effect as p53 to some extent. Indeed, our data show that overexpression of ECK in MCF-7 cells causes spontaneous apoptosis. The evidence for the role of ECK in tumorigenesis is limited and controversial. It was reported that activation of ECK/EphA2 receptor tyrosine kinase blocks the Ras/mitogen-activated protein kinase cascade in a variety of cell types and inhibits cell proliferation (23).

### Table 1. List of the Genes with the Palindrome in the Promoters

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<th>DIST</th>
<th>Description</th>
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<td>26118</td>
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<td>SOCS-box – containing WD protein SWIP-1</td>
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NOTE: The names of genes containing the palindrome motif (GTGACGTCAC) are listed with the indicated locus ID, distance from the transcriptional start site, and brief description. Abbreviations: LOCID, locus ID; DIST, distance from the transcriptional start site.
and cancer cell lines (24). It was reported that ectopic expression of ECK/EphA2 causes tumor progression of an immortalized breast cell line MCF-10F, and that the transformation capacity of ECK is related to the failure for ECK to interact with its ligands (24). Interestingly, stimulation of endogenous ECK in the same cell line reverses the tumor growth of the ectopic ECK-transformed cells (24). We found that the point mutation in the extracellular domain of ECK cripples ECK, revealing a link between tyrosine at codon 87 and ECK function as a cell death receptor. In addition, the mutation of the ligand-binding domain of ECK may affect the interaction between ECK and its ligand Ephrin-A1 for its functional properties. Ephrin-A1 was originally cloned as a cellular immediate-early response gene induced by cytokines, named B61 (25), and later was identified as the ligand for ECK (26). B61/Ephrin-A1 encodes a secreted protein that is markedly induced by TNF-α, a well-defined proapoptotic gene (27). We have recently observed that the levels of B61/Ephrin-A1 transcript and protein are much lower in many types of human cancer cell lines examined. Because ECK is activated by its ligands and functions to induce cell death, ECK would be an ideal target of cancer chemotherapeutics for the induction of apoptosis in cancer cells. Given the fact that p53 is defective in most cancer, this is significant because the activation of ECK in the absence of p53 may still induce spontaneous cell death. Because it is located at the cell membrane, ECK is easily accessible and targeted by chemical compounds.

**Materials and Methods**

Genomic Cloning and Sequence of the Human ECK Promoter

A human genomic DNA library constructed in the cosmid vector pWE15 (Clontech) was screened for the regulatory region upstream of the ECK using a 32P-labeled ECK cDNA fragment flanking the 5'-untranslated region and partial coding region as a probe, according to the manufacturer’s protocol. The primers used to amplify the ECK cDNA fragment from a human cDNA library by PCR are 5'-GATCGGACCGAGAGC-GAGAA-3' (forward) and 5'-CACGGAGTACATGTA-GATCGG-3' (reverse). Resulting positive clones from the human cDNA library were digested with restriction enzymes and subcloned into a pT-Adv vector (Clontech) for sequencing. One clone containing a 2.3-kb sequence of the regulatory region and first exon of the ECK gene was double sequenced using a series of primers. The Genbank accession number for the promoter of ECK is AY052403.

Cell Culture and DNA Transfections

EB and EB-1 have been described previously (8). H1299/p53ER cells were described elsewhere (9, 28). All cancer cell lines were from the American Type Culture Collection (Rockville, MD). These cell lines were maintained in Earl’s MEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO2. LipofectAMINE reagent (Life Technologies) was used for transient and stable transfection of cells. In a six-well culture plate, 3 × 10^5 cells were seeded per well and incubated for 24 hours. For each transfection, 1 to 2 μg of DNA were added into 100 μL serum-free medium, mixed with 25 μL of LipofectAMINE reagent, and incubated for 15 minutes. The solution was added directly onto the cells in 2-mL serum-free medium. The cells were incubated in MEM plus 20% fetal bovine serum for 16 hours. For stable clones, transfected cells were

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5 Liu and Yin, unpublished data.
grown in a complete growth medium containing 400 μg/mL G418 for neo resistance, or 2 μg/mL hygromycin B for hygromycin resistance for about 2 weeks. Colonies were picked up for further studies.

Construction of Luciferase Reporters and ECK Expression Plasmids

DNA fragments of the regulatory region of the ECK gene were amplified by PCR using corresponding primers. The primers used to amplify the whole ECK promoter (730 bp, –882 to –153) by PCR are as follows: 5’-GAAGCAGTGGTCAAGCAGAGACACCAGG-3’ (forward) and 5’-ACCGACTCCTGGATCATCTGCTGGTGGT (reverse). PCR reactions were done under the following conditions: seven cycles of 94°C for 2 seconds and 72°C for 3 minutes, 35 cycles of 94°C for 2 seconds and 67°C for 3 minutes, and one cycle of 67°C for 4 minutes. PCR products were subcloned into a pT-Adv vector (Clontech) for sequencing using T7 and M13 primers. The inserts were digested with appropriate restriction enzymes, purified, and subcloned into the pGL3-basic reporter (Promega). The single mutations of the pGL3/ECKp-0.7, pGL3/ECKp-0.7 m1, and pGL3/ECKp-0.7 m2 were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer’s protocol. The mismatched primers used to create point mutations in the palindromic site are M1, 5’-GGCGGGCTGGTGGCGTACGCAGGGC-3’ (forward) and M2, 5’-GGCGTTGGTACGGCAGGCAGGAGCATG-3’ (forward). All mutants were sequenced to confirm that only the designed point mutations were introduced. The ECK expression vector was constructed based on a selectable constitutive expression vector pcDNA3.1 (Invitrogen). The full coding region of human ECK was amplified from a human testis cDNA library (Clontech) by PCR using the following primers: hECK-up, 5’-ATCGGACCGAGAGCGAGAAG-3’; hECK-down, 5’-CTCGTAGATGCTCTCGTCGATGAGCT-3’. The resulting PCR product (3,133 bp) was ligated into the pT-Adv vector and then subcloned into the pGL3.1 vector at the sites of HindIII and XhoI, resulting in the pcDNA3.1/hECK. The mismatched primers used for generation of mutated ECK at nucleotide 373 are ECK-A/G373-forward, 5’-CGCGACCACTGCTGGTGGCCGAGGAGGAGC-3’ and ECK-A/G373-reverse, 5’-GCCACCGCCCTCCCTCGCCACACCCAGTTGGTGCGG-3’. All mutants were sequenced to confirm that only the designed point mutations were introduced.

Luciferase Assay

Cells were transiently transfected with plasmids in triplicate. Luciferase reporter containing p53-binding consensus sites in the promoter of p21 has been described previously (15, 16, 29). A pCMV-β-gal reporter plasmid was cotransfected to normalize the transfection efficiency. Cell extracts were processed using the Dual-Light kit (Applied Biosystems, Bedford, MA) according to the manufacturer’s instructions. Luciferase activity was measured with a Berthold Autolumat LB953 Rack Luminometer. Luciferase values were normalized against β-galactosidase activity. Luciferase readout was obtained from triplicate transfections and averaged.

RNA Isolation and Northern Blot

Total RNA was isolated from growing cells using TRIzol Reagent (Life Technologies). Poly(A)RNA was purified using a PolyATtract mRNA isolation system (Promega), according to the instructions of the manufacturers. For Northern analysis, 2 μg mRNA was run on a 1.2% formaldehyde gel and transferred to a Nybond-N membrane using a Turbo blotter system (Schleicher & Schuell, Florham Park, NJ). DNA probes were labeled with [α-32P]dCTP (Amersham Biosciences, Piscataway, NJ) using the Prime-It RmT Random Primer Labeling kit (Stratagene). The membrane was hybridized with labeled DNA probes in the QuikHyb hybridization (Stratagene) at 65°C for 2 hours and developed for autoradiograph.

Cellular Viability and Terminal Deoxynucleotidyl Transferase–Mediated Nick-End Labeling Assay

Cultured cells were seeded at an equal density of cells (2 × 105 per 100-mm dish) 1 day before experiments. The growing cells were treated in the indicated conditions and scored for numbers of viable and nonviable cells by trypan blue dye exclusion. For terminal deoxynucleotidyl transferase–mediated nick-end labeling assay, cells were cultured exponentially on tissue culture chamber slides. After treatment, the slides were fixed with 4% formaldehyde for 30 minutes. The slides were incubated with biotinylated dUTP and terminal transferase (Roche Diagnostics Corporation, Indianapolis, IN) in TDT buffer [30 mmol/L trizma base (pH 7.2) 140 mmol/L Na cacodylate, and 1 mmol/L cobalt chloride] for 1 hour. The slides were incubated with avidin-peroxidase and developed by Immuno Staining kit (Vector Laboratories, Burlingame, CA). Spontaneous apoptosis in MCF-7 clones was assayed using Fluorescein In situ Apoptosis Detection kit (Intergen, Purchase, NJ).

Immunoprecipitation and Western Blotting

Growing cells at 60% to 70% confluence were lysed in cold NP40 buffer with protease/phosphatase inhibitors. The precleared samples were immunoprecipitated with primary antibodies. Immunocomplexes were collected with protein A-Sepharose. Beads were washed with NP40 lysis buffer, resolved by 7.5% SDS-polyacrylamide gels, and then transferred onto nitrocellulose filter after protein separation. Immunoblots were incubated with primary antibodies and then incubated with peroxidase-conjugated rabbit anti-mouse IgG as secondary antibody. The signals were detected with enhanced chemiluminescence (Amersham).

Electrophoretic Mobility Shift Assay

Synthetic oligonucleotides (pairs of sense and antisense) were annealed and labeled with 32P by using T4 polynucleotide kinase and [γ-32P]ATP as described elsewhere (30). Recombinant p53 protein was produced in insect cells infected with a baculovirus vector expressing human wild-type p53 and partially purified through affinity chromatography. 32P-labeled probes (2 × 104 cpm) were mixed with 0.5 to 1 μg purified recombinant p53 or 5 μg nuclear extracts in a 20-μL DNA-binding reaction buffer consisting of 20 mmol/L Tris-HCl (pH 7.5), 4% Ficoll-400, 2 mmol/L EDTA,
0.5 mmol/L DTT, and 0.2 mg of poly(deoxyinosinic-deoxycytidylic acid). For specificity or competition controls, a labeled random oligonucleotide and excess of unlabeled corresponding oligonucleotide were added together in reactions. In some reactions, the anti-p53 monoclonal antibodies (PAb421/PAb1801, Oncogene Research Products, Uniondale, NY) were included for supershift. The reaction mixtures were incubated at 4°C for 20 minutes, resolved by a 4% polyacrylamide gel, and exposed to X-Omat film for photography.

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References

Molecular Cancer Research

A Novel Mechanism for p53 to Regulate Its Target Gene ECK in Signaling Apoptosis


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