p53 Stimulates Promoter Activity of the sgk Serum/Glucocorticoid-Inducible Serine/Threonine Protein Kinase Gene in Rodent Mammary Epithelial Cells*

(Revised for publication, January 11, 1996, and in revised form, March 22, 1996)

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sgk is a novel member of the serine/threonine protein kinase gene family that is transcriptionally regulated by serum and glucocorticoids in mammary epithelial cells. To functionally determine if the sgk promoter is regulated by the p53 tumor suppressor protein in mammary cells, a series of sgk promoter fragments with 5′-deletions were linked to the bacterial chloramphenicol acetyltransferase gene (sgk-CAT) and transiently cotransfected into nontumorigenic NMuMG or transformed ConA166 mammary epithelial cells with p53 expression plasmids. Wild-type p53, but not mutant p53, strongly stimulated sgk promoter activity in both mammary epithelial cell lines. These effects were mediated by specific regions within the sgk promoter containing p53 DNA-binding sites. The sgk p53 sequence at −1380 to −1345 (site IV) was sufficient to confer p53-dependent transactivation to a heterologous promoter, and p53 was capable of binding to this sequence in vitro as assessed by gel shift analysis. In the nontumorigenic NMuMG epithelial cell line, cotransfection of wild-type p53 strongly stimulated the activities of both the sgk promoter and the well characterized p53-responsive p21/Waf1 promoter, whereas in Rat-2 fibroblasts, wild-type p53 repressed the basal activities of both promoters, revealing that sgk and p21/Waf1 are similarly regulated in a cell type-specific manner. Taken together, these results demonstrate that sgk is a new transcriptional target of p53 in mammary epithelial cells and represent the first example of a hormone-regulated protein kinase gene with a functionally defined p53 promoter recognition element.

An intricate network of protein kinases and phosphatases propagates various extracellular growth and differentiation signals from the plasma membrane into the nucleus, leading to changes in the phosphorylation status and the function of discrete sets of transcription factors. The catalytic activities of most protein kinases are regulated by specific interactions with regulatory proteins (1–3) and/or by phosphorylation (4, 5). Recent studies have uncovered a newly emerging subfamily of serine/threonine protein kinase genes, including snk, sgk, plk, and fnk, that are predominantly regulated at the transcriptional level by hormone- and/or mitogen-induced pathways (6–13). Our previous studies have identified the sgk (serum- and glucocorticoid-inducible protein kinase) serine/threonine protein kinase gene, which is transcriptionally regulated by serum and/or glucocorticoids in mammary epithelial cells and Rat-2 fibroblasts (12, 13), as the second member of this subfamily of transcriptionally regulated protein kinase genes. sgk encodes a 49-kDa putative protein kinase that shares 45–55% homology with the catalytic domain of protein kinase C, the cAMP-dependent protein kinase A, the rac protein kinases, and the ribosomal protein S6 kinase (13). We have documented that sgk transcripts are expressed in a variety of adult rat tissues, with the highest expression in the thymus, lung, and ovary and detectable levels in the mammary gland and several other tissues (13). The cellular and tissue context strongly influences the expression of sgk since, depending on the cell type, different extracellular stimuli affect sgk transcription, or the same signals can regulate sgk expression with different kinetics. For example, in Rat-2 fibroblasts, induction of sgk gene transcription is an immediate-early response to serum that returns to near basal levels ~4 h after serum stimulation, whereas in mammary epithelial cells, sgk transcript levels remain at the induced levels for at least 48 h after their rapid induction by serum (12, 13). In granulosa cells of the rat ovary, sgk expression is regulated by a combination of testosterone and follicle-stimulating hormone (14), while in rat brain tissue, sgk expression is induced following injury to the central nervous system (15).

The cellular mechanisms governing the expression of sgk by diverse sets of extracellular stimuli are not well understood. To define the molecular details of this process and to uncover the transcriptional regulatory factors involved in modulating sgk gene expression, 4 kb of the sgk promoter region upstream of the transcriptional start site was cloned from a rat genomic library (13). Sequence analysis of the sgk promoter region revealed a glucocorticoid response element (GRE), at approximately −1.0 kb, that by functional analysis is responsible for the glucocorticoid-stimulated transcription of sgk (13). The sgk promoter also contains a TATA box and Sp-1 elements as well as putative binding sites for a variety of transcriptional regulators such as the AP-1 complex, CCAAT/enhancer-binding protein, NF-κB, GATA, and Ets-2,2 which in other systems have been found to be important for transducing proliferation.

1 The abbreviations used are: kb, kilobase(s); GRE, glucocorticoid response element; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]rsbq]glycine; bp, base pair(s).
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and/or differentiation signals (16–20). A striking feature of the sgk promoter is the presence of multiple putative binding sites for the p53 tumor suppressor protein, which implicates the sgk gene as a direct transcriptional target of p53. The p53 protein is a transcriptional regulator (21, 22) that plays an important role in cell cycle control, cellular differentiation, apoptosis, genomic stability, and response to DNA damage (23–26).

The p53 nuclear phosphoprotein can either positively or negatively regulate transcription in a gene- and tissue-specific manner. For example, the promoters of a variety of cellular genes associated with diverse p53-mediated responses are transcriptionally activated by p53 through p53-responsive elements in their promoter regions (27–38). In contrast, p53 represses transcription of several genes that lack p53-responsive elements (39–47). This repression response requires both the amino and carboxyl termini of p53 (48) as well as the oligomerization domain (49) and is likely due to interactions with components of core transcriptional machinery (44, 50, 51). Although not well characterized, for a few genes, transcriptional repression by p53 may involve its specific DNA binding capacity (42, 45). Additionally, p53 has also been shown to regulate activity of certain genes either positively or negatively depending on specific cell type (27, 52), suggesting the involvement of cell-specific factors or coregulators in p53-mediated transcriptional regulation.

In rat Con8Hd6 mammary tumor epithelial cells, from which the sgk protein kinase gene was cloned, treatment with glucocorticoids and serum suppresses cell growth and stimulates the cell-specific factors or coregulators in p53-mediated transcriptional components of core transcriptional machinery (44, 50, 51). Although not well characterized, for a few genes, transcriptional repression by p53 may involve its specific DNA binding capacity (42, 45). Additionally, p53 has also been shown to regulate activity of certain genes either positively or negatively depending on specific cell type (27, 52), suggesting the involvement of cell-specific factors or coregulators in p53-mediated transcriptional regulation. However, it is tempting to consider that sgk transcription may be regulated by p53. Besides the Con8Hd6 mammary tumor cells, sgk transcripts are also expressed and induced by glucocorticoids in nontumorigenic NMuMG mammary epithelial cells (13), thus providing a transformed (Con8Hd6) and a nontumorigenic (NMuMG) mammary epithelial cell line to compare the effects of p53. In this study, we show that p53 transactivates the sgk promoter in both NMuMG and Con8Hd6 mammary epithelial cells via a p53-responsive element located within the sgk promoter, thus demonstrating for the first time that a hormone-responsive protein kinase gene is a direct transcriptional target of the p53 tumor suppressor protein.

**EXPERIMENTAL PROCEDURES**

**Cells and Materials—**NMuMG nontransformed mouse mammary epithelial cells were originally derived from normal glandular tissue of an adult NAMRU mouse (54). Cells were regularly cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10 μg/ml insulin, and gentamicin sulfate. Rat-2 fibroblasts and rat Con8Hd6 mammary epithelial tumor cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium containing 10% calf serum. Cells were propagated at 37 °C in humidified air containing 5% CO₂ and the media were changed every 48 h. Dexamethasone was added to a final concentration of 1 μg/ml. Cultured cells were harvested at 40–48 h post-transfection, washed twice in ice-cold PBS, and then lysed in 1× reporter lysis buffer (Promega). The total amount of DNA used in CaPO₄ transfections for CAT assays was held constant at 20 μg, and in appropriate transfections, the total DNA was adjusted to this amount using the empty CAT vector plasmid pGL3 basic. For luciferase assays, cells were plated in 60-mm tissue culture plates, and a total of 4 μg of DNA (0.8 μg of WWP-Luc and 3.2 μg of either wild-type or mutant p53-encoding plasmids) were used for transfections. Con8Hd6 mammary tumor cells and Rat-2 fibroblasts were transfected by electroporation using previously described procedures (13). Typically, cells were transfected with 10 μg of reporter plasmid and 10 μg of the appropriate expression plasmid, with the total amount of DNA adjusted to 30 μg using the promoterless pBL-CAT vector DNA. Wherever indicated, the cell cultures were treated with 1 μM dexamethasone for 24 h prior to harvesting the cells. Transfections were performed in triplicates and repeated at least three times.

CAT and Luciferase Reporter Gene Assays—At 40–48 h post-transfection, for CAT and luciferase assays, the protein content of the cell extracts was estimated with the Bradford procedure (60). A quantitative nonchromatographic assay (61) was used to measure CAT activity in the cell extracts as detailed elsewhere (13). Cell extracts for luciferase assays were prepared by lysing the cells with 200 μl of reporter lysis buffer (Promega) according to the manufacturer’s instructions. A unit of reporter gene activity is defined as the amount of recombinant luciferase activity produced when injecting 100 μl of reconstituted luciferase assay reagent (Promega; 20 μM Tricine, 1.07 mM Mg(OAc)₂, 50 mM NaOH, 0.267 mM MgSO₄ 0.1 mM EDTA, 33.3 mM diethiothreitol, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP, pH 7.8) and mixing with 20 μl of cell extracts at room temperature. The light produced was measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

**Cell Morphology Shift Assays—**Preparation of nuclear extracts from NMuMG mammary cells was based on the method of Dignam et al. (62). The protein contents in the nuclear extracts were determined by the...
Transfection of Wild-type p53 Stimulates the Activity of the sgk Gene Promoter in NMuMG and Con8Hd6 Mammary Epithelial Cells—Transcription of the sgk gene is highly induced by the synthetic glucocorticoid dexamethasone both in Con8Hd6 mammary tumor epithelial cells and in nontumorigenic NMuMG mammary epithelial cells. This response is due to a functional GRE located at −1.0 kb within the sgk promoter (13).3 Sequence analysis of the sgk promoter has further revealed several putative binding sites for the p53 protein within the 1500-bp fragment just upstream of the transcription initiation site. To test if the sgk promoter is a transcriptional target of the p53 protein, nontumorigenic NMuMG and transformed Con8Hd6 mammary epithelial cells were transfected with a sgk promoter-CAT chimeric reporter plasmid containing −1428 bp of sgk promoter sequences fused to the CAT reporter gene (−1428sgk-CAT) alone or with expression plasmid encoding murine wild-type p53 protein. This sgk promoter fragment contains the GRE as well as four putative p53-binding sites. Transfected cells were treated with or without dexamethasone for 24 h, and cell extracts were assayed for CAT-specific activity. As shown in Fig. 1, dexamethasone stimulated the activity of the −1428sgk-CAT reporter plasmid in both NMuMG and

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Con8Hd6 mammary cells. Cotransfection of wild-type p53 elicited a 40-fold stimulation of the activity of the −1428sgk-CAT reporter plasmid in nontumorigenic NMuMG cells (Fig. 1, upper panel). The absolute level of sgk promoter activity was significantly increased in both dexamethasone-treated and untreated NMuMG cells transfected with wild-type p53. Furthermore, dexamethasone induced a mild increase in sgk promoter activity in the presence of wild-type p53. In Con8Hd6 mammary tumor cells, wild-type p53 stimulated a 6-fold increase in basal CAT activity that was approximately equal to the level of the induced promoter activity observed in cells treated with dexamethasone alone (Fig. 1, lower panel). No further increase in sgk promoter activity was observed in dexamethasone-treated Con8Hd6 cells transfected with wild-type p53.

Parallel sets of mammary cells were transfected with a GRE-CAT reporter plasmid that contains six copies of GREs fused upstream of the CAT reporter gene, or with the CMV-CAT reporter plasmid, which is known to be transcriptionally repressed by p53 in a variety of cell types (46). As shown in Fig. 2, dexamethasone strongly stimulated GRE-CAT activity in both cell types (left panel), while cotransfection of wild-type p53 strongly repressed CMV-CAT reporter gene activity (right panel) under conditions in which p53 induced sgk promoter activity. Taken together, our results show that wild-type p53 strongly activates the glucocorticoid-responsive sgk promoter in both nontumorigenic (NMuMG) and transformed (Con8Hd6) mammary epithelial cells in a dexamethasone-independent manner.

Mapping of p53-responsive Regions within the sgk Promoter by Deletion Analysis—To determine the region of the sgk promoter that confers the responsiveness to wild-type p53, a series of 5′-progressive deletions in the sgk promoter were generated by controlled exonuclease III digestion. The corresponding sgk promoter-CAT chimeric reporter plasmids (sgk-CAT) were constructed to contain varying lengths of sgk promoter sequences that all terminate at +51 in the sgk gene. The resulting sgk-CAT constructs had four (−1428sgk-CAT), two (−681sgk-CAT, −303sgk-CAT), one (−236sgk-CAT), or no (−190sgk-CAT) putative p53 DNA-binding sites in the sgk promoter region (Fig. 3A, lower panel). NMuMG or Con8Hd6 cells were cotransfected with the sgk-CAT reporter plasmids alone or together with expression vectors for either murine wild-type p53 or mutant p53 with two point mutations at amino acid residues 168 and 234, which renders the mutant deficient in DNA binding (64).

Analysis of CAT-specific activity revealed that cotransfection of wild-type p53 caused a 23-fold stimulation of the promoter activity of −1428sgk-CAT in NMuMG mammary epithelial cells, whereas a significant reduction in the extent of induction by wild-type p53 was observed in the sgk-CAT constructs containing progressively shorter sgk promoter fragments (Fig. 3B). Transfection of the mutant p53 expression plasmid had no effect on sgk promoter fragments except for a mild stimulatory effect on the reporter plasmid containing the −1428-bp sgk fragment. A similar pattern of activation only by wild-type p53, but not mutant p53, was observed in transfected Con8Hd6 mammary tumor cells, although the magnitude of induction of sgk-CAT deletion constructs by wild-type p53 was less compared with that of NMuMG cells (Fig. 3B). The p53-mediated stimulation of sgk promoter activity in both nontumorigenic NMuMG and transformed Con8Hd6 mammary cells appeared to target a similar region of the sgk 5′-upstream sequence that contains two p53 DNA-binding sites between −681 and −1428 of the sgk promoter (p53 sites III and IV) likely to be responsible for most of the p53 response.

sgk p53 DNA-binding Site IV Is Sufficient to Confer Positive Transcriptional Effects of p53 to a Heterologous Promoter—Of the two putative p53-binding sites in the p53-responsive region of the sgk promoter, p53 site IV between −1380 and −1345 bp is most homologous to the consensus p53-binding site. To test if this p53 recognition site alone is sufficient to render the positive transcriptional regulation observed with the sgk promoter to a heterologous promoter, an oligonucleotide corresponding to sgk p53 site IV was inserted into a CAT vector upstream of sequence −105 to +50 of the thymidine kinase minimal promoter to form sgk p53tk-CAT. NMuMG mammary cells were cotransfected with either a sgk p53tk-CAT or a tk-CAT reporter plasmid that does not contain p53 site IV sequences along with expression vectors for either wild-type or mutant p53. Cells were also transfected with two other reporter plasmids, PG13-CAT, which contains 13 copies of DNA sequences that bind p53 in vitro (55) fused upstream of the CAT reporter gene, or MG15-CAT, which contains 15 copies of mutated p53-binding sequence. These reporter plasmids were either transfected alone or cotransfected with either wild-type or mutant p53 encoding plasmids. Measurement of CAT activity demonstrated that wild-type p53 stimulated, by ~8-fold, the activity of the sgk p53tk-CAT reporter plasmid in NMuMG epithelial cells, while mutant p53 had no effect on sgk p53tk-CAT activity.
Fig. 3. p53-mediated activation of sgk promoter-CAT deletions in Con8Hd6 and NMuMG cells. A, the upper panel shows the sequences of the four putative p53 recognition elements within the −1428-bp sgk promoter fragment and are designated as sites I–IV. The base pairs underlined denote nucleotides sharing homology with the p53 consensus sequence. The lower panel illustrates the location of the putative p53-binding sites and the GRE in the context of the −1428sgk-CAT reporter construct. The arrows and corresponding numbers refer to the 5′-end points of the sgk promoter deletions incorporated into the −1428sgk-CAT, −681sgk-CAT, −303sgk-CAT, −236sgk-CAT, and −190sgk-CAT reporter plasmids. B, the indicated sgk-CAT reporter plasmids (10 μg) were transfected alone or cotransfected with 10 μg of expression vectors for either wild-type p53 (wt p53) or mutant p53 (mt p53) into either Con8Hd6 cells (left panel) or NMuMG cells (right panel) as described in the text. The 5′-sgk promoter fragments used are shown on the far right, and the corresponding numbers refer to the 5′-end points of deletions of the various constructs. Forty hours post-transfection, cells were assayed for CAT activity as described for Fig. 1, and the CAT activity was normalized to protein levels. Data represent means ± S.D. obtained from at least three separate transfections, each carried out in triplicate.

(Fig. 4). Transfection of the minimal promoter-containing tk-CAT reporter plasmid only resulted in a minor stimulation by wild-type p53 in NMuMG cells while remaining at basal levels under all other conditions. As expected, cotransfection of wild-type p53 stimulated, up to 5-fold, the activity of wild-type p53-responsive PG13-CAT reporter plasmid, but not the mutant MG15-CAT reporter plasmid (Fig. 4). Mutant p53 had negligible effects on both of the reporter plasmids. These results demonstrate that the putative p53 site IV in the sgk promoter is a functional p53 recognition element that can confer positive transcriptional regulation of sgk promoter activity by wild-type p53 in normal NMuMG epithelial cells.

Interaction of p53 with the sgk p53 Site IV Sequence by Gel Shift Analysis—Since the 35-bp p53 site IV sequence present between −1380 and −1345 bp of the sgk promoter is sensitive to transcriptional regulation by p53, gel shift experiments were utilized to examine the formation of nuclear protein-DNA complexes. Nuclear extracts isolated from NMuMG epithelial cells were incubated with a 32P-labeled oligonucleotide corresponding to −1380 to −1345 bp of the sgk promoter, and protein-DNA complexes were resolved by native polyacrylamide gel electrophoresis. Incubation of nuclear extracts derived from the NMuMG epithelial cells with the sgk p53 DNA probe (site IV) resulted in the formation of three distinct protein-DNA complexes, A, B, and C (Fig. 5, left panel). To determine the specificity of these interactions, a series of competition experiments were carried out using different types of unlabeled oligonucleotides. The specific complexes were completely eliminated by the addition of a 100-fold molar excess of specific unlabeled sgk p53 site IV DNA, but not by same amount of nonspecific unrelated DNA (nonspecific DNA) or mutated consensus p53-binding site DNA (Mut p53). When excess unlabeled DNA corresponding to the p53 consensus sequence (Con p53) was used as a competitor, only protein-DNA complex C was efficiently competed off.
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Fig. 5 Gel shift analysis of binding of p53 with the sgk p53 site IV sequence. Nuclear extracts (10 μg) prepared from NMuMG cells were incubated with 32P-end-labeled double-stranded DNA probes representing region 1380 to 1345 of the sgk promoter (site IV). The indicated reaction mixtures contained no extract; no unlabeled competitor DNA (No competitor); a 100-fold molar excess of double-stranded oligonucleotides corresponding to sgk p53 site IV, the consensus p53-binding site (Con p53); an unrelated DNA (nonspecific DNA), or a mutant p53-binding site (Mut p53); or a 100-fold molar excess of unlabeled competitor DNA sgk p53 site I (–235 to –205), sgk p53 site II (–285 to –255), or sgk p53 site III (–1155 to –1125). Binding reactions were preincubated for 1 h with 100 ng of anti-p53 antibodies (PAb421). The protein-DNA complexes formed were separated by 4% native polyacrylamide gel electrophoresis and visualized by autoradiography. Arrows indicate the positions of protein-DNA complexes and free probe.

Fig. 6 p53 present in nuclear extracts as determined by Western blotting interacts with p53 consensus sequences in NMuMG cells as assessed by gel shift analysis. Nuclear extracts (10 μg) prepared from NMuMG cells were incubated with 32P-end-labeled double-stranded DNA probes representing the p53 consensus sequence (Con p53). The indicated reaction mixtures contained no extract; no unlabeled competitor DNA (No competitor); or a 100-fold molar excess of double-stranded oligonucleotides corresponding to sgk p53 site IV, the consensus p53-binding site (Con p53), an unrelated DNA (nonspecific DNA), or a mutant p53-binding site (Mut p53). Binding reactions were preincubated for 15 or 60 min with 100 ng of anti-p53 antibodies (PAb421). The protein-DNA complexes formed were separated by 4% native polyacrylamide gel electrophoresis and visualized by autoradiography. Arrows indicate the positions of protein-DNA complexes and free probe. Nuclear protein extracts (30 μg) from NMuMG cells were electrophoretically fractionated, blotted onto nitrocellulose filters, and analyzed for p53 protein by Western blotting as described under “Experimental Procedures” (inset). The protein molecular mass standards are shown on the left. The arrow indicates the position of the p53-specific band.

while the two slower migrating complexes, A and B, were not affected (Fig. 5, left panel). The specificity of binding of p53 with the sgk p53 site IV sequence was also examined by preincubating the nuclear extracts for 1 h with the PAb421 anti-p53 monodonal antibody. The p53 monoclonal antibody disrupted the formation of protein-DNA complex C (Fig. 5, left panel, PAb421). In addition to the sgk p53 site IV sequence, three other putative p53-binding sites designated as site I (–235 to –205), site II (–285 to –255), and site III (–1155 to –1125) exist within the sgk promoter that differ from each other and from the p53 consensus sequence to varying extents. Competition experiments were carried out to test if the different sgk p53 sequences can specifically compete for p53 binding with the radiolabeled site IV DNA probe. As also shown in Fig. 5 (right panel), the specific complex formed (No competitor) can be competed off with a 100-fold molar excess of unlabeled sgk p53 sequences spanning site III, II, or I, but not with an unrelated DNA sequence (nonspecific DNA). Gel shift analysis using NMuMG nuclear extracts and radiolabeled oligonucleotides corresponding to sgk p53 sites I–III revealed the specific formation of protein-DNA complex C, as observed with site IV, with each putative sgk p53-binding site, although the protein binding appeared to be weaker compared with that of site IV (data not shown).

To functionally test for the presence of p53 in nuclear extracts, a parallel set of gel shift experiments was carried out using a radiolabeled oligonucleotide corresponding to the consensus p53 DNA-binding site. In contrast to the three protein-DNA complexes detected with the sgk DNA site IV probe in NMuMG extracts (Fig. 5), incubation of the p53 consensus probe with these nuclear extracts yielded a single specific complex (Fig. 6, No competitor). Competition experiments established the specificity of binding. For example, the addition of excess specific unlabeled Con p53 fragment completely abolished the single band, whereas excess unrelated nonspecific DNA or DNA encoding a mutated p53-binding site was incapable of competing off the protein-DNA complex (Fig. 6). More importantly, the specific complex using radiolabeled Con p53 was competed off with excess unlabeled sgk p53 site IV DNA. When the nuclear extracts were preincubated with p53 antibodies for a short time (15 min), a partial supershift of the specific complex was observed, whereas upon longer incubation (60 min) with p53-specific antibodies, a completely supershifted complex was obtained. The presence of p53 in these nuclear extracts was analyzed by Western blotting. As also shown in Fig. 6, detectable amounts of p53 were present in these nuclear extracts, accounting for the specific protein-DNA complex formation. These results show that p53 present in the nuclear extracts of NMuMG epithelial cells can bind specifically to the sgk p53 site IV sequence or the consensus p53 DNA-binding site. In addition, the different putative sgk p53-binding sequences (sites III, II, and I) are all homologous to some extent to the site IV sequence as these three sites can efficiently compete for specific binding of p53 with the radiolabeled sgk p53 site IV DNA probe.

Cell Type-specific Activation or Repression of the sgk Promoter by Wild-type p53 Protein—Previous studies have shown that the magnitude and kinetics of sgk gene expression can be differentially regulated in epithelial cells versus fibroblasts (12). Since p53 has been shown to differentially regulate target promoters in a cell type-specific manner (27, 52), the cell type-specific transcriptional effects of wild-type p53 protein on sgk...
for gene expression is rapidly stimulated (13), implicating a role of part of the target genes. Our results demonstrate that, by p53 further supports this idea and represents the first example of a hormone-regulated protein kinase gene that contains a functionally defined p53-responsive element in its promoter region.

Deletion analysis of the sgk promoter revealed that the p53-responsive region observed in both transfected mammary epithelial cell lines harbors two putative p53-binding sites, sgk p53 site I (−1380 to −1345) and sgk p53 site II (−1155 to −1125). Transfection assays demonstrated that the 35-bp fragment corresponding to sgk p53 site I alone was functional and that p53 specifically interacted with these sequences in vitro. We have also observed specific binding of p53 to the sgk p53 site II sequence (data not shown). Previous studies have defined two copies of the 10-bp motif 5′-RRRC(A/T)(A/T)GYYY-3′ separated by 0–13 bp (63) as the p53 consensus sequence. The sgk p53 site I sequence (5′-ctGCGGACTGCTGTCCCC-3′) as well as the site II sequence (5′- gagGGGCGGACATGCAGGGTAATTGACTG-3′) exhibit strong homology to the p53 consensus sequence compared with the other two p53-binding sites, I and II, outside of the functionally defined p53-responsive region. These two sites may account for the minor activation of sgk-CAT reporter plasmids containing shorter regions of sgk promoter activity in transfected NMuMG cells. A wide range of degeneracy in p53 recognition sequences exists as a variety of p53-activated promoters contain p53-binding sites different from the p53 consensus sequence. For instance, the p53-responsive element within the human T-cell leukemia virus type I enhancer is composed of a GC-rich element (5′-GCCCTGACTGCTGTCCCC-3′) (65). Other p53-responsive elements that differ from the consensus sequence include the two copies of the weak p53-binding element (5′-GGGCGGAGTTA-3′) within the SV40 origin of replication (66) and the p53 sequences defined from mouse genomic clones (5′-GACACTGCGGACTGCTGTCCCC-3′) (67). In this regard, although sgk p53 site I and II sequences are not strongly related to the p53 consensus sequence, p53 was capable of specifically binding to these sequences in vitro (data not shown), and these sequences efficiently competed with the sgk p53 site I sequence for binding as assessed by gel shift assays.

In contrast to the strong p53-dependent stimulation of sgk promoter activity in mammary cells, wild-type p53 evoked a repression of sgk promoter activity in Rat-2 fibroblasts. More importantly, wild-type p53 regulated the activity of the p21/Waf1 gene promoter, a well characterized transcriptional target of p53 (38), in a cell type-specific manner generally similar to the effects observed on sgk promoter activity. This result further establishes the sgk gene as a biologically relevant transcriptional target of p53. The exact mechanism involved in the p53-mediated down-regulation of cellular promoters is not well understood, although it has been proposed that repression by
p53 occurs by interaction with components of the general transcriptional machinery (44, 50, 51) and, in some cases, may entail binding of p53 to specific DNA sequences (42, 45). In fact, the cell type-specific positive or negative regulation of genes by p53 appears to be confined to genes containing p53-binding sites. For example, the creatine kinase gene and proliferating cell nuclear antigen gene promoters, which contain functional p53-binding sites within their promoter regions, are also either positively or negatively regulated in a cell type-specific manner (27, 29, 52). In contrast, a CMV-CAT reporter that lacks p53-binding sites is and is known to be repressed by p53 (46) was suppressed in p53 by both mammary epithelial cells and Rat-2 fibroblasts (data not shown). A variety of viral and cellular proteins (68, 69) and also certain transcription factors (70) have been shown to interact with p53 and to modulate its function (71–74). Thus, depending on the cellular context, p53 is able to affect either transcription or repression of the sgk gene, or the p21 gene, presumably in conjunction with cell type-specific regulatory factors.

Many transcriptional target genes of p53 are involved in important cellular processes such as growth regulation (e.g., fos, jun, myc, cyclin A, and p21/Waf1) (38, 40, 75, 76) and apoptosis (e.g., bcl-2 and bax) (33, 42). The cell type-specific growth suppression effects of p53 appear to be related to the ability of p53 to act as a specific transcriptional activator and, in certain tissues, its ability to interact with the MDM2 protein (77). Many studies have implicated p53 to be important for the growth control of mammary epithelial cells. For example, approximately one-fourth of all sporadic breast cancers express mutant forms of p53 containing deletions or mutations (78), whereas other mammary tumors are characterized as having an allelic loss of the short arm of chromosome 17, which includes p53 (79, 80). Consistent with a role for p53 in the evolution of mammary tumors, a deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice (81). Other evidence suggests that p53 mutations are important in the early preneoplastic stages of mammary tumorigenesis (77). In addition, loss of p53 protein in certain types of mammary cells exposed to γ-irradiation (53) or as a result of human papillomavirus E6-induced degradation (82) is accompanied by immortality of these cells and failure to arrest in the G1 phase of the cell cycle.

In our study, wild-type p53, but not mutant p53, strongly stimulated sgk promoter activity in both nontumorigenic and transformed mammary epithelial cells. In this context, functional characterization of a positive p53 recognition element within the promoter of this protein kinase gene implies that a subset of p53-mediated responses in mammary cells, such as on the control of cell proliferation or programmed cell death after exposure to DNA-damaging agents (83), may be mediated by positive or negative changes in cellular phosphorylation cascades and/or activities of potential sgk substrates. In mammary tumor epithelial cells, we propose that sgk may be involved in the glucocorticoid-regulated growth suppression response and that activation of sgk by p53 may represent a distinct, but complementary, growth regulatory pathway. A key focus of our future approaches will be to understand the functional roles and biological context by which p53 targets the sgk gene in mammary epithelial cells and if activation of the sgk promoter requires mammary-specific nuclear factors or coactivators to interact with the p53-DNA complex.

Acknowledgments—We express sincere thanks to Zrin Cram, Yukihiro Nishio, Ross A. Ramos, and Paul Woo for critical comments on this manuscript. We also thank Jerry Kaplan for skillful photography and Charles Jackson, William J. Meilant, Marina Chin, Althaea Yrowode, Khanh Tong, Vinh Trinh, and Thai Truong for technical support.
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