THE GUT-ENRICHED KRÜPEL-LIKE FACTOR (KRÜPEL-LIKE FACTOR 4) MEDIATES THE TRANSACTIVATING EFFECT OF p53 ON THE p21^{WAF1/Cip1} PROMOTER

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Running Title: Transcctivation of the p21^{WAF1/Cip1} Promoter by p53 and GKLF

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SUMMARY

An important mechanism by which the tumor suppressor p53 maintains genomic stability is to induce cell cycle arrest through activation of the cyclin-dependent kinase inhibitor, p21<sup>WAF1/Cip1</sup>, gene. We show that the gene encoding gut-enriched Krüppel-like factor (GKLF; KLF4) is concurrently induced with p21<sup>WAF1/Cip1</sup> during serum deprivation and DNA damage elicited by methyl methane sulfonate (MMS). The increases in expression of both GKLF and p21<sup>WAF1/Cip1</sup> due to DNA damage are dependent on p53. Moreover, during the first 30 minutes of MMS treatment, the rise in GKLF mRNA level precedes that in p21<sup>WAF1/Cip1</sup>, suggesting that GKLF may be involved in the induction of p21<sup>WAF1/Cip1</sup>. Indeed, GKLF activates p21<sup>WAF1/Cip1</sup> through a specific Sp1-like cis-element in the proximal p21<sup>WAF1/Cip1</sup> promoter. The same element is also required by p53 to activate the p21<sup>WAF1/Cip1</sup> promoter, although p53 does not bind to it. Potential mechanisms by which p53 activates the p21<sup>WAF1/Cip1</sup> promoter include a physical interaction between p53 and GKLF, and the transcriptional induction of GKLF by p53. Consequently, the two transactivators cause a synergistic induction in the p21<sup>WAF1/Cip1</sup> promoter activity. The physiological relevance of GKLF in mediating p53-dependent induction of p21<sup>WAF1/Cip1</sup> is demonstrated by the ability of antisense GKLF oligonucleotides to block the production of p21<sup>WAF1/Cip1</sup> in response to p53 activation. These findings suggest that GKLF is an essential mediator of p53 in the transcriptional induction of p21<sup>WAF1/Cip1</sup> and may be part of a novel pathway by which cellular responses to stress are modulated.
INTRODUCTION

A principal function of the tumor suppressor p53 is to maintain genomic stability. It does so by eliciting cellular changes in response to various forms of stress such as DNA damage, hypoxia, and nucleotide deprivation (1-3). The amount of p53 protein increases in response to these so-called genotoxic stresses. In addition, covalent modifications such as phosphorylation are involved in its activation (4,5). Once activated, p53 exerts potent regulatory effects on diverse aspects of cellular events that cumulate in cell cycle arrest or apoptosis (3). Many of these “downstream” events are dependent upon the ability of p53 to function as a transcription factor in activating the expression of “target” genes (2,6). Notably, an important consequence of p53 activation is the transcriptional induction of the gene encoding the cyclin-dependent kinase (Cdk) inhibitor p21 (also called WAF1 or Cip1) (7,8). p21\textsuperscript{WAF1/Cip1} inhibits the activity of several cyclin-Cdk complexes such as cyclin D1-Cdk4, cyclin E1-Cdk2 and cyclin A-Cdk2, which results in cell cycle arrest at the G1-S transition checkpoint (9, 10).

The gut-enriched Krüppel-like factor (GKLF or KLF4; reference 11) is a recently identified and developmentally regulated transcription factor the expression of which is enriched in the epithelial cells of the gastrointestinal tract (12-14), skin (14,15), and thymus (16), and in vascular endothelial cells (17). Both the in vivo (12-16) and in vitro (12) patterns of expression of GKLF are indicative of a growth arrest-associated nature. Upon stimulation of quiescent cultured cells by fresh serum, levels of GKLF mRNA are decreased significantly during the G1-S transition phase of the cell cycle (12). Conversely, constitutive expression of GKLF inhibits DNA synthesis (12). In vivo, GKLF transcripts are highly enriched in the population of terminally differentiated, post-mitotic epithelial cells of the intestinal tract and skin (12-15).
Moreover, the intestinal expression of *GKLF* is down-regulated in two independent mouse models of intestinal tumorigenesis or hyperproliferation (18,19). Taken together, these studies suggest that GKLF is potentially a negative regulator of proliferation, however, the mechanism by which it accomplishes this task is not well defined.

The established binding site for GKLF is rich in GC content (20) and overlaps with that for the transcription factor Sp1 (21,22). By coincidence, the proximal promoter of the *p21WAF1/Cip1* gene contains a number of GC-rich elements (7), some of which have been shown to bind Sp1 (23–29). These Sp1-binding sites have been shown to be important in controlling expression of *p21WAF1/Cip1* in several physiologically diverse processes, including the gene’s responsiveness to phorbol ester (23), transforming growth factor-β (TGF-β) (24–26), and sodium butyrate (27), and in keratinocyte differentiation (28). As both *GKLF* and *p21WAF1/Cip1* are growth arrest-associated genes, we sought to determine whether GKLF is involved in regulating *p21WAF1/Cip1* expression. We demonstrate that GKLF not only transactivates the *p21WAF1/Cip1* proximal promoter but also mediates the activating effects of p53 in response to DNA damage on the same promoter. This study suggests that GKLF may be an important component of the p53 tumor suppressor network of regulatory proteins.
EXPERIMENTAL PROCEDURES

Plasmid constructs, reagents and cell lines

The eukaryotic expression vector PMT3 and its derivatives containing various forms of GKLF were previously described (12,20,30,31). They included full-length GKLF [PMT3-GKLF-(1-483)], truncated GKLF that lacked the three zinc fingers [PMT3-GKLF-(1-401)], and truncated GKLF that contained the zinc fingers only [PMT3-GKLF-(350-483)]. pC53-SN3 and pC53-SX3, two CMV-based expression constructs containing wild-type p53 and mutated p53 with a missense mutation at codon 143 (m143) in the DNA binding domain (DBD) of p53, respectively, were kindly provided by B. Vogelstein and K. Kinzler (32). The reporter constructs linking various regions of the p21WAF1/Cip1 promoter to chloramphenicol acetyl transferase (CAT) have previously been described (23). They included the CAT reporter linked to either 2,320 nt 5’-flanking sequence of the p21WAF1/Cip1 gene that contained an upstream p53-binding site at nt –2301 (33) or the same 2,320 nt 5’-flanking sequence with a small internal deletion of the sequence between nt -122 and -61 of the p21WAF1/Cip1 promoter which removed the first 4 of the 6 Sp1 sites from the proximal promoter (33). Reporter constructs containing the proximal promoter region of the p21WAF1/Cip1 gene with various 5’ endpoints as well as internal deletions or point mutations affecting the various Sp1 sites in the proximal promoters have all been described (23). The WWP-Luc and DM-Luc constructs were two luciferase reporters that contained 2.4 and 2.2 kb, respectively, of the 5’-flanking sequence of the p21WAF1/Cip1 gene and were kindly provided by Vogelstein and Kinzler (7). The DM-Luc construct lacked the upstream p53-binding site at nt –2301 in the p21WAF1/Cip1 promoter (7).
The polyclonal rabbit anti-GKLF serum was described (12). Anti-p53 serum was purchased from Santa Cruz (#sc-6243) and the monoclonal antibody against p21\textsuperscript{WAF1/Cip1} was purchased from Pharmingen (SXM30). The p53 -/- and +/+ mouse embryonic fibroblasts (MEFs) were generously provided by L. Donehower (34). The 10(1)-p53 val135 cell line was provided by A. Levine (35). This cell line, which was derived from the parental 10(1) cell line (36), is an immortalized murine embryo fibroblast line that lacked endogenous p53 expression but contained a stably transfected temperature-sensitive p53 protein, val135 (37). At the non-permissive temperature of 38.5 °C, p53 val135 is transcriptionally inactive; whereas at the permissive temperature of 31.5 °C, it is transcriptionally competent (35). The sense and antisense oligonucleotides to GKLF contained nucleotide sequences corresponding to aa codons 7 to 13 of GKLF in the sense and antisense orientation, respectively. At the center of this sequence, aa position 10, is the second of two initiation methionine codons of GKLF, which was felt to be in a translationally more favorable context than the first (14). The nucleotide sequence of the antisense oligonucleotide was 5'- GCT GAC AGC CAT GTC AGA CTC -3' and that of the sense oligonucleotide was 5'- GAG TCT GAC ATG GCT GTC AGC -3'. Note that the underlined sequence represents the initiation methionine codon at aa position 10 (12).

**Conditions of cell treatments, and Northern and Western blot analyses**

For the serum deprivation experiments, the content of fetal calf serum (FCS) in the cell media was reduced from 10% to 0.5% to induce a growth-arrested state (12). To cause DNA damage, methyl methane sulfonate (MMS) was added to cells at a concentration of 100 µg/ml, which has previously been shown to result in cell cycle arrest (38). After various treatment periods, total RNA was isolated from cells using Triazol (Life Technology). Twenty µg RNA
from each sample were studied by Northern blot analyses using conditions previously described (12). Blots were probed with a full-length cDNA encoding GKL (12), p21^{WAF1/Cip1} (7), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech). The conditions for Western blot analysis were also previously described, using a 1:1,000 dilution of an affinity-purified polyclonal anti-GKL serum (12).

**Transfection, luciferase and CAT assays**

All transfections were performed by lipofection as described (20,21,30,31). Unless otherwise specified, all reactions contained 5 µg/10-cm dish each of the reporter and effector constructs. Luciferase and CAT assays were performed as described (20,21).

**Reverse transcription-polymerase chain reactions (RT-PCR)**

RNA was extracted from human embryonic kidney (HEK) 293 cells and the human colonic carcinoma HT29 cells (39). The content of GKL transcript from each cell line was determined using RT-PCR. The content of the β-actin transcript was similarly determined as a control. One µg RNA was reverse transcribed in an 80 µl volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl$_2$, 0.5 mM each of dGTP, dATP, dTTP, and dCTP, 80 U RNase inhibitor, 100 pmol random primer pd(N)$_6$, and 200 U MMLV reverse transcriptase (Life Technology) at 42 °C for 1 hr. The cDNA was then amplified in a 50 µl reaction that contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl$_2$, 0.1% gelatin, 2.5 U REDTaq DNA polymerase (Sigma), 0.2 mM each of dGTP, dATP, dTTP, and dCTP, and 40 pM each of the forward and reverse primers (see below), at the following settings: 94 °C, 45 sec;
45 °C, 1 min; 72 °C, 1.5 min; for a total of 40 cycles. The PCR products were then visualized on a 1.5% agarose gel stained with ethidium bromide.

The primers used in the PCR reaction were synthesized according to the published cDNA sequences encoding human GKLF and β-actin (GeneBank Accession numbers AF105036 and X00351, respectively). The forward primer sequence for GKLF was 5’-AGGTCGGACCACCTCGCCTTACACATG-3’ and the reverse primer sequence was 5’-AAGGTAAAGAGAATACAAGGTGATCTTTTATGC-3’. The length of the expected PCR product was 345 bp. The forward and reverse primer sequences for β-actin were 5’-TACGCCAACACAGTGCTGTCTGG -3’ and 5’-TACTCCTGCTTGCTGATCCACAT -3’, respectively, with the expected PCR product measuring 206 bp.

Electrophoretic mobility shift assays

EMSAs were performed as described (20). Preparation of nuclear extracts from COS-1 cells transfected with PMT3 expression constructs containing full-length (FL) GKLF, truncated GKLF containing only the zinc fingers (ZF) or lacking the zinc fingers (∆ZF), or PMT3 vector alone was previously described (20,21). Purified p53 containing the DNA-binding domain (DBD) was kindly provided by N. Pavletich (40). This domain contained the core portion of p53 between aa residues 102-292, which bound with high affinity to a p53-recognition site (40,41). The purification of recombinant p53 DBD expressed from the pET3d bacterial expression vector (Novagen) in transformed E. coli BL21(D3) cells was previously described (40). The protein was supplied at a concentration of 14 mg/ml in a solution of 50 mM bistrispropane-HCl, pH 6.8, 200 mM Na phosphate, and 5 mM DTT and had a >98% purity of the core domain.
The wild-type p21 oligonucleotide used in EMSA contained the sequence between nt –129 and –99 of the p21\textsuperscript{WAF1/Cip1} promoter, which included both Sp1-1 and Sp1-2 sites (27). The mutant p21 oligonucleotide contained a 3-bp substitution in the Sp1-1 site. The sequences in the sense orientation for the two oligonucleotides are shown below:

Wild-type 5’- GCCCGGGGAAGGCGGTTCCCGGGCGGC -3’

\[\frac{\text{Sp1-1}}{\text{Sp1-2}}\]

Mutant 5’- GCCCGGGGAAGATGGTCCCGGGCGGC -3’

\[\frac{\text{mutation}}{\text{Sp1-2}}\]

The oligonucleotide probe containing the binding site for p53 was derived from the p53-response sequence in the promoter of the human GADD45 gene (42) and had the sequence 5’-TACAGAACATGTCTAAGCATGCTGGG -3’ in the sense orientation. When indicated, unlabeled competitor oligonucleotides were added in 10-, 20- or 50-fold molar-excess of the probe to the reaction.

\textit{In vitro synthesis of p53 and immunoprecipitation}

\[^{35}\text{S}]\text{methionine-labeled p53 was synthesized by the TNT Coupled Reticulocyte Lysate System (Promega) using a full-length cDNA encoding p53 cloned in pBluescript (provided by B. Vogelstein). Ten \mu l of the translation product was mixed with 50 \mu g of nuclear extracts prepared from transfected COS-1 cells in a final volume of 100 \mu l containing 20 mM HEPES, pH 7.5, 40 mM KCl, 3 mM MgCl\textsubscript{2}, 1 mM DTT, and 5\% glycerol at 4 °C for 2 hr. At the completion of the incubation, 15 \mu g affinity-purified anti-GKLF serum or pre-immune serum were added to the reaction which was gently rotated overnight at 4 °C. Fifty \mu l packed protein A-Sepharose beads}
(Pharmacia) were then added to each reaction and the incubation continued for 1 hr at 4 °C. The beads were subsequently collected by centrifugation, washed three times with the incubation buffer, and resuspended in sample buffer before electrophoresis.
RESULTS

Both GKLF and p21\(^{WAF1/Cip1}\) are induced during growth arrest

Previously, we showed that the levels of the GKLF transcript were low in actively proliferating cells but were increased in cells that had been deprived of serum (12). Results of the Northern blot analysis in Figure 1A recapitulate this event. Figure 1A also shows that upon serum deprivation, the levels of the \(p21^{WAF1/Cip1}\) transcript rose concomitantly with those of GKLF. To determine whether GKLF is induced during growth arrest under a different condition, we treated NIH 3T3 cells with methyl methane sulfonate (MMS), which causes DNA damage and subsequently cell cycle arrest (38). As seen in Figure 1B, the levels of GKLF mRNA were increased 2 hr after the addition of MMS, as were those of \(p21^{WAF1/Cip1}\) mRNA. When normalized to the expression of the control GAPDH gene, which was not affected by the treatment, the degree of induction in \(p21^{WAF1/Cip1}\) was higher (between 1.6- and 2.8-fold) than that in GKLF between 2 and 8 hr of MMS treatment (Figure 1B, bar graph). This contrasts with the changes in mRNA levels of the two genes during the initial 30 min of treatment, in which the rise in GKLF preceded that in \(p21^{WAF1/Cip1}\) (Figure 1C). These results suggest that both GKLF and \(p21^{WAF1/Cip1}\) respond similarly to signals elicited during growth arrest due to DNA damage. However, the induction of GKLF begins slightly earlier than that of \(p21^{WAF1/Cip1}\) during the initial phase of DNA damage.

The induction of GKLF and \(p21^{WAF1/Cip1}\) by MMS is dependent on p53

To determine whether the inductive responses of GKLF and \(p21^{WAF1/Cip1}\) to MMS treatment are dependent on p53, we compared the expression of the two genes in mouse embryonic fibroblasts (MEFs) isolated from mice that contained (\(p53^{+/+}\)) and lacked \(p53^{−/−}\) (p53 -
/-) created by homologous recombination (34). As seen in Figure 2, neither fibroblasts contained appreciable amounts of GKLF and p21$^{WAF1/Cip1}$ in the untreated state, despite a relative abundance of p53 in the $p53^{+/+}$ cells (lanes 1 and 2). Upon the addition of MMS, there was a dramatic increase in the levels of GKLF and p21$^{WAF1/Cip1}$ beginning at 2 hr but only in $p53^{+/+}$ MEFs (lanes 4, 6, and 8). In contrast, although there was a p53-independent response in GKLF and p21$^{WAF1/Cip1}$ production to MMS in $p53^{-/-}$ cells (lanes 3, 5 and 7), this component appeared minor compared to the $p53$-proficient cells. We conclude that the increase in expression of $GKLF$ in response to MMS-induced DNA damage, like p21$^{WAF1/Cip1}$, is dependent on the presence of p53.

*Both GKLF and p53 transactivate the p21$^{WAF1/Cip1}$ proximal promoter through an identical cis-element*

The sequential pattern of expression in $GKLF$ followed by p21$^{WAF1/Cip1}$ immediately after the addition of MMS raised the intriguing question whether GKLF might be responsible in part for the induction of p21$^{WAF1/Cip1}$. We considered this plausible as the promoter of the p21$^{WAF1/Cip1}$ gene contains a number of GC-rich cis-elements that resemble Sp1-binding sites (7, 23-29) and GKLF has been shown to bind to a GC-rich DNA sequence with which Sp1 also interacts (20,21).

To determine whether GKLF regulates the p21$^{WAF1/Cip1}$ promoter, we performed co-transfection experiments in human embryonic kidney (HEK) 293 cells using a series of p21$^{WAF1/Cip1}$ promoter-reporter constructs (23) and an expression construct containing either the wild-type or a mutated GKLF that had its zinc fingers deleted (Figure 3A, effectors 2 and 3,
respectively) (12,20,30,31). Since p53 has been shown to transcriptionally activate a reporter gene when linked to the 5’ flanking sequence of \( p21^{WAF1/Cip1} \) (7,23), expression constructs containing the wild-type or a mutated p53 that lost its ability to bind DNA (Figure 3A, effectors 4 and 5, respectively) (32) were also included in the analysis. Consistent with a previous report (13), HEK 293 cells contained a negligible amount of \( GKLF \) transcript at baseline level as determined by RT-PCR, relative to a human colon cancer carcinoma cell line, HT29 (Figure 3L). This low level of baseline \( GKLF \) expression in HEK 293 cells allowed a better delineation of the effects of GKLF on the \( p21^{WAF1/Cip1} \) promoter activity.

Figure 3B shows that both wild-type GKLF and p53 (effectors 2 and 4, respectively), but not mutated GKLF and p53 (effectors 3 and 5, respectively), transactivated the CAT reporter gene linked to nt -2,320 to +16 of the \( p21^{WAF1/Cip1} \) promoter sequence. However, neither wild-type GKLF nor wild-type p53 was able to transactivate the same promoter that had a small internal deletion in the sequence between nt –122 and –61 (Figure 3C). These results indicate that GKLF, like p53, is capable of activating the \( p21^{WAF1/Cip1} \) promoter and that in order for both proteins to act on the promoter, the sequence between nt –122 and –61 is essential. The dependence of p53 on this proximal region of the \( p21^{WAF1/Cip1} \) promoter was unexpected since the binding sites for p53 in 2,320 nt of the promoter were previously localized to sequences much further upstream from the immediate flanking region of the \( p21^{WAF1/Cip1} \) gene (43).

The sequence between nt –122 and –61 of the \( p21^{WAF1/Cip1} \) promoter contains 4 GC-rich elements that resemble Sp1-binding sites, which have previously been designated Sp1-1 to Sp1-4 sites in the 5’ to 3’ direction (27). To precisely define the \( cis \)-element(s) within this sequence
that mediates the activating effect of GKLF and p53 on the \( p21^{\text{WAF1/Cip1}} \) promoter, we performed additional co-transfection experiments in which the CAT reporter gene was linked to either –154 to +16 bp of the promoter (Figure 3D) or to one that contained various 5' and internal deletions, or point mutations (Figures 3E-3K). It is clear from the results of these experiments that the transactivating effects of GKLF and p53 were co-localized to an identical cis-element, which was the first Sp1-binding site (Sp1-1 site) beginning at nt –116 in the \( p21^{\text{WAF1/Cip1}} \) promoter.

**GKLF but not p53 binds to the Sp1-1 element in the \( p21^{\text{WAF1/Cip1}} \) promoter**

To determine whether GKLF or p53 binds to the Sp1-1 sequence identified above, we performed electrophoretic mobility shift assays (EMSAs) between GKLF and a labeled oligonucleotide containing the sequence between nt –129 and –99 of the \( p21^{\text{WAF1/Cip1}} \) promoter [\( p21^{(\text{wt})} \) Probe; Figure 4A], or between the DNA-binding core domain (DBD) of p53 (40,41) and an established p53-binding sequence (\( p53 \) Probe; Figure 4B). Nuclear extracts prepared from COS-1 cells transfected with the PMT3 expression vector containing either full-length (FL) GKLF (Figure 4A, lane 1) or the zinc finger (ZF) portion of GKLF (Figure 4A, lane 9) bound to the \( p21^{(\text{wt})} \) probe. The resulting DNA-protein complexes (C1 or C2) were competed away by an unlabeled wild-type \( p21^{\text{WAF1/Cip1}} \) sequence (Figure 4A, lanes 2-4 and 10-12, respectively) but not by a mutated competitor in which the Sp1-1 site was destroyed due to a 3-bp substitution (Figure 4A, lanes 5-7 and 13-15, respectively). As controls, nuclear extracts prepared from either vector alone-transfected cells (C; lane 16) or cells transfected with a mutant GKLF construct that lacked the zinc fingers (\( \Delta ZF \); lane 17) did not exhibit any appreciable binding to the \( p21^{(\text{wt})} \) probe. The results in Figure 4A therefore provide strong evidence that GKLF interacts directly with the Sp1-1 site of the \( p21^{\text{WAF1/Cip1}} \) promoter. In contrast, the DNA-binding
domain of p53, although clearly capable of binding to an established p53-binding sequence (Figure 4B, lane 2), failed to interact with the $p21^{WAF1/Cip1}$ sequence since an unlabeled wild-type $p21$ probe did not compete at all (Figure 4B, lanes 6-8). Moreover, p53 DBD failed to form a complex with a labeled $p21$ (wt) oligonucleotide (data not shown). These results suggest that although the transactivating effect of p53 on the $p21^{WAF1/Cip1}$ proximal promoter depends on the Sp1-1 site, as does GKLF, it is mediated by a mechanism that does not involve the direct binding of p53 to the DNA.

$p53$ physically interacts with GKLF and transcriptionally activates the GKLF promoter, resulting in a synergistic activation of the $p21^{WAF1/Cip1}$ promoter by p53 and GKLF

One potential method by which p53 may accomplish its indirect effect on the $p21^{WAF1/Cip1}$ proximal promoter is by forming a physical complex with GKLF, thus gaining access to the promoter. To test this hypothesis, we performed co-immunoprecipitation experiments using in vitro synthesized p53 and GKLF produced in transfected cells. As shown in Figure 4C, anti-GKLF serum ($\alpha$) specifically co-precipitated p53 when p53 was combined with nuclear extracts from cells transfected with either the zinc finger region of GKLF (Figure 4C, lane 6) or full-length GKLF (Figure 4C, lane 7), but not with those transfected with the PMT3 vector alone (Figure 4C, lane 8). No p53 was detected in any of the reactions incubated with pre-immune (PI) serum (Figure 4C, lanes 3-5). These findings provide strong evidence for a physical interaction between p53 and GKLF in a region that includes the zinc fingers of GKLF. It is of interest to note that only full-length p53 but not an internally initiated p53 with an estimated molecular weight of 40 kDa (Figure 4C, lane 1) was recovered in the immunoprecipitates (Figure 4C, lanes...
6 and 7). This suggests that the N-terminal portion of p53 may be necessary for the interaction with GKLFL.

The dependence of GKLFL induction on p53 as seen in Figure 2 suggests that GKLFL, like p21WAF1/Cip1, is regulated by p53 during DNA damage. Indeed, the results in Figure 4D show that p53 transcriptionally activates GKLFL since a luciferase reporter linked to 5.0 (Figure 4D, lane 1) but not 1.0 kb (Figure 4D, lane 3) of the mouse GKLFL promoter was transactivated by wild-type 53. In contrast, a mutated p53 failed to activate either reporter (Figure 4D, lanes 2 and 4). The degree of induction in the –5.0 kb GKLFL promoter activity by p53 was comparable to that seen for 2.4 kb of the p21WAF1/Cip1 promoter (p21 WWP-Luc; Figure 4D, compare lanes 1 and 5). These results, therefore, suggest that there is a p53-response element(s) between –5.0 and –1.0 kb of the GKLFL promoter. The exact location of this element(s) has not been determined since only the first kb of the GKLFL promoter has been sequenced so far (22) and does not contain a p53-binding site.

To determine whether the physical interaction between GKLFL and p53 and the transcriptional induction of GKLFL by p53 are physiologically relevant to the regulation of the p21WAF1/Cip1 promoter, we performed co-transfection experiments using sub-saturating concentrations of expression vectors containing GKLFL, p53, or both, and a luciferase reporter gene containing 2.4 (WWP-Luc) or 2.2 kb (DM-Luc) of the p21WAF1/Cip1 promoter sequence (7). The two reporters differed from each other in that WWP-Luc included an upstream p53-binding site located at nt –2,301 (33). As shown in Figure 5, the combination of GKLFL and p53 resulted in a synergistic induction of the p21WAF1/Cip1 promoter either in the presence (lane 3) or absence
These results suggest that GKLF and p53 act in a cooperative manner to activate \( p21^{WAF1/Cip1} \) gene expression by a mechanism that does not require the upstream p53-binding site of the \( p21^{WAF1/Cip1} \) promoter.

**GKLF is necessary for the inductive effect of p53 on the \( p21^{WAF1/Cip1} \) promoter**

The sequential induction of \( GKLF \) and \( p21^{WAF1/Cip1} \) during the early phase of DNA damage and the physical dependence of p53 on GKLF in activating the proximal \( p21^{WAF1/Cip1} \) promoter raised the possibility that GKLF may be important in mediating the effect of p53 on stimulating \( p21^{WAF1/Cip1} \) gene expression. To address this possibility, we examined a system in which activation of p53 is inducible due to a temperature sensitive mutation. As shown in Figure 6A, induction of wild-type p53 activity by shifting 10(1) cells stably transfected with the temperature sensitive p53 val135 (35,37) from the non-permissive (38.5 °C) to permissive (31.5 °C) temperature resulted in a considerable accumulation of GKLF as well as \( p21^{WAF1/Cip1} \) to a degree similar to that observed in MMS-treated \( p53^{+/+} \) MEFs (Figure 2). Next, to assess the role of GKLF in mediating the inductive effect of p53 on \( p21^{WAF1/Cip1} \) expression, we treated cells with a 21-nt antisense oligonucleotide containing the sequence that surrounds the initiation methionine codon of GKLF in an attempt to block the translation of \( GKLF \) mRNA. We then determined the effects of such treatments on the production of \( p21^{WAF1/Cip1} \) at the permissive temperature. A sense oligonucleotide with the complementary sequence to the antisense oligonucleotide was used as a control. As shown in Figure 6B, cells treated with increasing concentrations of the antisense oligonucleotide contained progressively lower levels of GKLF (Figure 6B, lanes 3, 5, and 7). Importantly, the same cells produced correspondingly lower levels of \( p21^{WAF1/Cip1} \) as well. Although there was some decrease in the levels of GKLF, and
consequently p21^{WAF1/Cip1}, in cells treated with the highest concentration (3.0 µM) of the control sense oligonucleotide (Figure 6B, lane 6), this was probably due to a non-specific, perhaps toxic side-effect of the high oligonucleotide concentration. Be that as it may, the results in Figure 6B indicate that a decreased production of GKLF leads to an inhibition of p21^{WAF1/Cip1} synthesis. We, therefore, conclude that GKLF is an important mediator of the action of p53 in inducing p21^{WAF1/Cip1} gene expression.
DISCUSSION

The present study reveals a novel mechanism by which expression of the p21\(^{WAF1/Cip1}\) gene is modulated during cellular stress induced by DNA damage. At least five lines of evidence in the study suggest that GKLF plays a physiologically relevant and possibly crucial role in mediating the activating effect of p53 on p21\(^{WAF1/Cip1}\) expression: (1) the sequential manner in which GKLF and p21\(^{WAF1/Cip1}\) are expressed in the immediate period following DNA damage (Figure 1C), (2) the requirement of the GKLF-response element in the p21\(^{WAF1/Cip1}\) promoter (i.e. the Sp1-1 site) for the transactivating effect of p53, despite the presence of other \textit{bona fide} p53-response elements in the same promoter (Figures 3B and 3C), (3) the physical interaction between p53 and GKLF (Figure 4C) and the transcriptional induction of \textit{GKLF} by p53 (Figure 4D), (4) the cooperative manner in which GKLF and p53 activate the p21\(^{WAF1/Cip1}\) promoter (Figure 5), and (5) the ability of antisense GKLF oligonucleotides to inhibit p21\(^{WAF1/Cip1}\) synthesis upon p53 activation (Figure 6). These findings demonstrate that p53 may depend on GKLF to activate the p21\(^{WAF1/Cip1}\) promoter, thus implicating GKLF as an important component of the p53 network of cell cycle regulators.

Based on the observations of this study, we propose a model which portrays the regulation of the proximal p21\(^{WAF1/Cip1}\) promoter by GKLF and p53 during cellular stress elicited by DNA damage. In this model, activation of p53 represents an immediate response to DNA damage as depicted by numerous previous studies (reviewed in 1-3). The activated p53 causes an increase in the quantity of GKLF, which is mediated, at least in part, at the level of transcription (Figure 4D). In addition, p53 physically interacts with GKLF and consequently allows it to gain access to the proximal p21\(^{WAF1/Cip1}\) promoter through the Sp1-1 site to which
GKLF alone binds. A result of this complex relationship is the synergistic induction in the activity of the $p21^{\text{WAF1/Cip1}}$ promoter (Figure 5). This model, therefore, would predict an immediate and possibly maximal induction in expression of the gene encoding $p21^{\text{WAF1/Cip1}}$ following DNA damage. This would assure the immediate cessation of cell cycle progression due to the potent inhibitory effect of $p21^{\text{WAF1/Cip1}}$ on cyclin-dependent kinases (48). It is of note that despite the involvement of the various Sp1 elements in the $p21^{\text{WAF1/Cip1}}$ promoter in mediating the responses of the promoter to numerous other physiological stimuli (Figure 7), the lone utilization of the Sp1-1 site by GKLF and consequently by p53 has not previously been documented.

The mechanism by which GKLF participates in the regulation of the $p21^{\text{WAF1/Cip1}}$ promoter by p53 is reminiscent of that for another growth arrest-associated gene, $GADD45$ (49). Like $GKLF$ and $p21^{\text{WAF1/Cip1}}$, expression of $GADD45$ is induced by genotoxic stresses such as DNA damage (50). In addition to a strong p53-binding element in an intronic sequence of $GADD45$ (51), p53 was shown to contribute to the stress response of the $GADD45$ promoter (50). Much of this stress responsiveness was localized to a GC-rich motif of the proximal promoter to which the tumor suppressor, Wilm’s Tumor 1 (WT1; 52) binds, but p53 does not. The mechanism by which p53 activates the promoter is thought to be mediated by its ability to physically interact with WT1 (50). This resulted in a strong and cooperative induction of the $GADD45$ promoter when p53 and WT1 were concurrently introduced (50). Finally, abrogation of WT1 function by an antisense vector markedly reduced the induction of the $GADD45$ promoter (50). Similar to the conclusion of the present study, the authors concluded that p53
contributes to the positive regulation of the *GADD45* promoter primarily by protein-protein interactions.

Recent literature provides another example in which the *p21WAF1/Cip1* promoter can be cooperatively regulated by multiple proteins with important functions in cell cycle control. In that study, BRCA1 was shown to transactivate the proximal *p21WAF1/Cip1* promoter through the region between nt –117 and –93, which contains both Sp1-1 and Sp1-2 sites (45). This resulted in an inhibition of progression into the S-phase in cells that over-expressed BRCA1 (45). Importantly, p53 potentiated the BRCA1-dependent activation of the *p21WAF1/Cip1* promoter by physically interacting with BRCA1 (53). Thus, it appears that p53 activates expression of its target genes such as *p21WAF1/Cip1* and *GADD45*, by multiple but perhaps interrelated mechanisms. These mechanisms include direct binding of p53 to the classical p53-response elements and indirect interaction with non-consensus binding sites through physical contacts with other regulatory proteins, including GKLF, WT1, and BRCA1.

Another potential mechanism responsible for the synergistic induction of the *p21WAF1/Cip1* promoter by p53 and GKLF may involve the participation of other regulatory proteins. In this regard, both p53 (54,55) and GKLF (31) have been shown to interact with a group of transcriptional co-activators, including p300 and CBP (56-59). In fact, the ability of GKLF to activate transcription is dependent on its interaction with p300/CBP (31). Thus, it is possible to modify the model proposed in Figure 7 to include p300/CBP, which can serve as a bridge between p53/GKLF and the basal transcriptional machinery such as the TATA-binding factor and RNA polymerase II (60,61). It is of interest to note that p300/CBP are enzymes that display...
histone acetylase activity (62,63) and that the activity of the $p21^{WAF1/Cip1}$ promoter is subject to regulation by compounds that alter chromatin structure due to acetylation such as butyrate, trichostatin A (TSA), and trapoxin (TPX) (Figure 7). Moreover, the Sp1-like cis-elements responsible for the action of these compounds appear to differ among one another (Figure 7). It is formally possible that the targets of regulation by these compounds may be unique transcription factors that recognize the different Sp1-like elements in the $p21^{WAF1/Cip1}$ promoter.

Results in Figure 1 indicate that both $GKLF$ and $p21^{WAF1/Cip1}$ are induced by serum deprivation and by DNA damage. The kinetics in which the two genes are induced by the two conditions, however, are distinctly different from each other. During the course of serum deprivation, the extent of induction for both $GKLF$ and $p21^{WAF1/Cip1}$ is quite similar (Figure 1A). However, the time-course of induction for $GKLF$ and $p21^{WAF1/Cip1}$ in cells treated with MMS is different from that of serum deprivation. Thus, with the exception of an earlier induction of $GKLF$ during the initial 30 min of MMS treatment (Figure 1C), the level of induction of $p21^{WAF1/Cip1}$ after 1 hr of MMS treatment is significantly higher than that of $GKLF$ (Figures 1B and 1C). These results suggest that factors in addition to GKLF may be involved in the rise in $p21^{WAF1/Cip1}$ transcript level after the immediate phase of DNA damage. However, the parallel rise in the levels of both $GKLF$ and $p21^{WAF1/Cip1}$ transcript during serum deprivation suggest a potentially more uniform mechanism of induction of the two genes, perhaps including a mechanism that is independent of p53 as has been demonstrated in other systems (64). Experiments are in progress to address this potentially p53-independent component of $GKLF$ and $p21^{WAF1/Cip1}$ activation.
In the intestinal tract, *GKLF* transcript is detected primarily in the terminally differentiated, post-mitotic epithelial cells (12-14). Interestingly, *p21^{WAF1/Cip1}* transcript is also distributed in the same cell population (65). Moreover, the intestinal expression of *p21^{WAF1/Cip1}* both during development and in the adult mouse has been shown to be independent of p53 at basal condition (66). Whether the *in vivo* expression of *GKLF* is also independent of p53 is unclear at this point. However, it is clear that the induction of both *GKLF* and *p21^{WAF1/Cip1}* in response to genotoxic stress are highly dependent on p53 (Figure 2). Moreover, this inductive response is not limited to the intestinal cell lineage and includes fibroblasts such as NIH 3T3 and MEFs. Thus, the *in vitro* behavior of *GKLF* as modulated by stress is more ubiquitous than its *in vivo* tissue distribution. This may be viewed as an additional evidence for the potentially broader significance of GKLF in mediating the “guardian” function of p53.
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ABBREVIATIONS
CAT, chloramphenicol acetyl transferase; Cdk, cyclin-dependent kinase; DBD, DNA-binding domain; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; FL, full-length; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GKLF, gut-enriched Krüppel-like factor; KLF4, Krüppel-like factor 4; Luc, luciferase; MEF, mouse embryonic fibroblast; MMS, methyl methane sulfonate; RT-PCR, reverse transcription-polymerase chain reaction; ts, temperature sensitive; ZF, zinc finger.
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FIGURE LEGENDS

Figure 1. Northern blot analysis of GKLF and p21WAF1/Cip1 in NIH 3T3 cells during growth arrest

Growth arrest was induced in actively proliferating NIH 3T3 cells maintained in a medium containing 10% fetal calf serum (FCS) by the reduction of serum content to 0.5% (panel A) or by the addition of 100 µg/ml methyl methane sulfonate (MMS) to the media (panels B and C). RNA was isolated at the indicated time points and 20 µg were loaded in each lane and analyzed for the message content of GKLF, p21WAF1/Cip1, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The lower portion of the figure contains the quantitative information of fold-induction in GKLF (open bars) and p21WAF1/Cip1 (closed bars) at each treatment time point over the untreated (basal) value for each experiment. The calculation was performed first by normalizing the band intensity of the GKLF or p21WAF1/Cip1 transcript to that of GAPDH at each time point and then comparing the normalized value of GKLF or p21WAF1/Cip1 at each treatment time point to that of untreated cells (time 0).

Figure 2. Western blot analysis of GKLF and p21WAF1/Cip1 in mouse embryonic fibroblasts (MEFs) proficient or deficient in p53

MEFs were prepared from p53-deficient (-/-) mouse embryos (34) or their wild-type littermate control (+/+), and treated with 100 µg/ml MMS for the time periods indicated. Proteins were isolated and analyzed for the content of p53, GKLF, or p21WAF1/Cip1 by Western blot analysis. Load represents a portion of the gel stained with Commassie blue before electrophoretic transfer.
**Figure 3.** GKLF and p53 transactivate the p21WAF1/Cip1 promoter

Panel A depicts the five effectors used throughout the co-transfection studies. Effector 1 is the PMT3 expression vector alone. Effectors 2 and 4 are expression constructs of wild-type GKLF and p53, respectively. Effector 3 is a mutated GKLF that does not have its zinc fingers (ZF) (30). Effector 5 is a mutated p53 with a missense mutation at codon 143 (X) in the DNA-binding domain (DBD) of p53 (32). Various regions of the p21WAF1/Cip1 promoter were linked to the chloramphenicol acetyl transferase (CAT) reporter (panels B-K) and co-transfected with an equivalent quantity of the various effectors into HEK 293 cells. The 4 Sp1-binding sites (27) between nt –122 and –61 of the promoter are represented by the four arrowheads. The locations for Sp1-1 and Sp1-2 are identified in panel D. The X in panels J and K represent a 3-bp mutation in the first (J) and second (K) Sp1-binding site, respectively. The numbers in the X-axis in panels D to K correspond to the 5 effectors shown in panel A. C is the substrate chloramphenicol and AC represents the acetylated product. % conversion equals AC/AC + C x 100. Shown in panels D to K are the means of three independent experiments. Bars represent standard deviations. Panel L is the result of RT-PCR of the mRNA levels of GKLF and β-actin in HT29 and HEK 293 cells.

**Figure 4.** The relationship among p53, GKLF, and the Sp1-1 site of the p21WAF1/Cip1 promoter

(A) GKLF binds to the Sp1-1 site. Electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts prepared from COS-1 cells transfected with an expressing construct that contains the full-length (FL) GKLF (lanes 1-7) or the zinc finger (ZF) region of GKLF (lanes 9-15) and a radiolabeled oligonucleotide probe containing the sequence between nt –129 and –99 of the p21WAF1/Cip1 promoter, which includes both Sp1-1 and Sp1-2 sites (27).
Where indicated, increasing amounts of unlabeled oligonucleotides representing either the wild-type (wt) sequence or a mutated (mut) sequence that contains a 3-bp substitution in the Sp1-1 site, were included. Lane 8 contains the probe alone without added proteins. Lanes 16 and 17 were performed with nuclear extracts obtained from COS-1 cells transfected with the PMT3 vector alone (C) and the GKLF construct that lacked the zinc fingers (ΔZF) as in Figure 3A, respectively. C1 is the complex formed between full-length GKLF and the probe, and C2 is formed between the zinc fingers and the probe. F is free probe. (B) p53 does not interact with the sequence between nt –129 and –99 of the p21WAF1/Cip1 promoter. EMSAs were performed with purified DNA-binding domain (DBD) of p53 (40) and a labeled probe representing an established p53-binding site. Competitors include unlabelled p53 binding sequence (lanes 3-5) and unlabelled wild-type p21WAF1/Cip1 sequence between nt –129 and –99 (lanes 6-8). C3 indicates the complex between p53 DBD and the probe. (C) GKLF interacts with p53. [35S]-labeled p53 synthesized by in vitro transcription and translation was mixed with nuclear extracts from COS-1 cells transfected with PMT3-GKLF (ZF) (zinc fingers), PMT3-GKLF (FL) (full-length), or PMT3 vector alone, and precipitated with either pre-immune (PI) serum or anti-GKLF (α) serum. Lane 1 (*) contains the input p53 and lane 2 is p53 mixed with protein A-Sepharose beads without added serum. The precipitated materials were resolved by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. (D) p53 transactivates the GKLF promoter. Either 5.0 or 1.0 kb of the 5’-flanking sequence of the mouse GKLF gene was linked to a luciferase reporter and co-transfected into Chinese hamster ovary (CHO) cells with an expression construct containing either wild-type (wt) or a mutated (mut) p53 that no longer binds DNA (see Figure 3A). Included was a p21 WWP-Luc construct that contained 2.4
kb of the \( p21^{WAF1/Cip1} \) promoter sequence linked to the luciferase reporter as a control (7). Shown are the means of 4 experiments. Bars are standard deviations.

**Figure 5.** Synergistic activation of the \( p21^{WAF1/Cip1} \) promoter by GKLF and p53

Co-transfection experiments were performed in HEK 293 cells with a luciferase reporter linked to 2.4 or 2.2 kb of the \( p21^{WAF1/Cip1} \) promoter sequence (WWP-Luc that contained an upstream p53-binding site at nt –2,301 or DM-Luc that did not, respectively; references 7 and 33) and sub-saturating amounts of expression constructs containing GKLF, p53, or both. Shown are the means of 4 independent experiments. Vertical bars represent standard deviations.

**Figure 6.** GKLF mediates the inductive effect of p53 on \( p21^{WAF1/Cip1} \)

(A) Induction of \( GKL\) and \( p21^{WAF1/Cip1} \) in 10(1) cells (36) containing a temperature-sensitive (ts) mutant p53 protein. 10(1) cells stably expressing the val135 ts p53 mutant (35,37) were propagated at either the non-permissive temperature of 38.5 °C or the permissive temperature of 31.5 °C for the time periods indicated. Proteins were harvested and analyzed for p53, GKLF, or \( p21^{WAF1/Cip1} \) by Western blot analysis. Both GKLF and \( p21^{WAF1/Cip1} \) were absent at time zero when cells were maintained at 38.5 °C (results not shown). (B) Inhibition of \( p21^{WAF1/Cip1} \) formation in the 10(1)-p53 val135 cell line by antisense oligonucleotides to GKLF. Cells were transfected by lipofection with increasing amounts of sense (S) or antisense (AS) oligonucleotides to GKLF at 38.5 °C for 5 hr and shifted to 31.5 °C for an additional 24 hr before being harvested for quantification of p53, GKLF, or \( p21^{WAF1/Cip1} \) by Western blot analysis.

**Figure 7.** A model for the regulation of the proximal \( p21^{WAF1/Cip1} \) promoter by p53 and GKLF
The locations of the 6 Sp1-like elements within –154 bp of the \( p21^{WAF1/Cip1} \) promoter are designated according to a previous report \( (27) \). The model illustrates that the activation of p53 by DNA damage leads to both an increase in GKLF synthesis and an interaction between p53 and GKLF (double arrow), which cumulates in the binding of GKLF to the Sp1-1 element of the \( p21^{WAF1/Cip1} \) promoter. The various Sp1 \( cis \)-elements that mediate the functions of other physiological stimuli are also indicated. They include the phorbol ester, PMA, and okadaic acid (OA) \( (23) \); trapoxin (TPX), a histone deacetylase inhibitor \( (44) \); BRCA1, the breast cancer tumor suppressor gene \( (45) \); transforming growth factor-\( \beta \) (TGF-\( \beta \)) \( (24) \); \( \text{Ca}^{++} \), which is important in keratinocyte differentiation \( (28) \); GGTI, a geranylgeranyltransferase I inhibitor \( (46) \); butyrate \( (27) \) and trichostatin A (TSA) \( (29) \), both also histone deacetylase inhibitors; levostatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor \( (47) \); and progesterone \( (43) \).
Fold induction

N = 4

|       | 1 | 2 | 3 | 4 | 5 | 6 |
|-------|---|---|---|---|---|---|
| WWP-Luc (μg) | 5 | 5 | 5 | 0 | 0 | 0 |
| DM-Luc (μg)   | 0 | 0 | 0 | 5 | 5 | 5 |
| GKL (μg)      | 2 | 2 | 2 | 0 | 2 | 2 |
| p53 (μg)      | 0 | 2 | 2 | 0 | 2 | 2 |
A

|       | 8       | 24      | 48 hr  |
|-------|---------|---------|--------|
| 38.5° | S       | S       | S      |
| 31.5° | AS      | AS      | AS     |

- p53
- GKLF
- p21

B

|       | 0       | 0.3     | 1.0    | 3.0    |
|-------|---------|---------|--------|--------|
| S     | S       | S       | S      |
| AS    | AS      | AS      | AS     |

- p53
- GKLF
- p21
The Gut-Enriched Kruppel-Like Factor (Kruppel-Like Factor 4) Mediates the Transactivating Effect of p53 on the p21WAF1/Cip1 Promoter
Weiqing Zhang, Deborah E. Geiman, Janiel M. Shields, Duyen T. Dang, Channing S. Mahatan, Klaus H. Kaestner, Joseph R. Biggs, Andrew S. Kraft and Vincent W. Yang

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