The Role of the Transcription Factor Sp1 in Regulating the Expression of the WAF1/CIP1 Gene in U937 Leukemic Cells*

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The WAF1/CIP1 protein induces cell cycle arrest through inhibition of the activity of cyclin-dependent kinases and proliferating cell nuclear antigen. Expression of the WAF1/CIP1 gene is induced in a p53-dependent manner in response to DNA damage but can also be induced in the absence of p53 by agents such as growth factors, phorbol esters, and okadaic acid. WAF1/CIP1 expression in U937 human leukemic cells is induced by both phorbol ester, a protein kinase C activator, and by okadaic acid, an inhibitor of phosphatases 1 and 2A. Both of these agents induce the differentiation of these leukemic cells toward macrophages. We demonstrate that phorbol esters and okadaic acid stimulate transcription from the WAF1/CIP1 promoter in U937 cells. This transcription is mediated by a region of the promoter between −154 and +16, which contains two binding sites for the transcription factor Sp1. Deletion or mutation of these Sp1 sites reduces WAF1/CIP1 promoter response to phorbol ester and okadaic acid, while a reporter gene under the control of a promoter containing only multiple Sp1 binding sites and a TATA box is induced by phorbol ester and okadaic acid. The WAF1/CIP1 promoter is also highly induced by exogenous Sp1 in the Sp1-deficient Drosophila Schneider SL2 cell line. These results suggest that phorbol ester and okadaic acid activate transcription of the WAF1/CIP1 promoter through a complex of proteins that includes Sp1 and basal transcription factors.

Treatment of the human myeloid leukemia cell line U937 with phorbol esters such as phorbol myristate acetate (PMA), † an activator of protein kinase C, leads to macrophage-monocyte-like differentiation over a 72-h period (1, 2). This process involves changes in cell-substrate adherence, growth arrest in late G1, and increased expression of monocyte markers (3, 4). Similarly, treatment of U937 cells with okadaic acid, a natural product isolated from the black sponge and a potent inhibitor of protein phosphatases 1 and 2A, also induces differentiation of these cells (5), cell cycle arrest, and eventual (72-h) apoptosis (6). Both PMA and okadaic acid induce expression of the cyclin-dependent kinase inhibitor, WAF1/CIP1 (7, 8).

WAF1/CIP1 expression is induced by the p53 protein following irradiation of cells (9, 10), but p53-independent expression of WAF1/CIP1 is associated with differentiation of myocytes (11, 12), of HL 60 leukemic cells (13), and of a number of other cells. WAF1/CIP1 is expressed in a number of tissues over the course of murine development, and expression in most tissues is not dependent on the presence of p53 (14). p53-independent expression of the WAF1/CIP1 gene can be induced in cultured cells by a number of agents including, besides PMA and okadaic acid, platelet-derived growth factor, fibroblast growth factor, and transforming growth factor β (15, 16).

Preliminary analysis of the WAF1/CIP1 promoter suggests that the elements mediating response to serum in fibroblasts are located at least 1.9 kb upstream from the transcription start site (14), while responsiveness to tumor growth factor β is mediated by elements located somewhere in the promoter sequences 1.3 kb upstream of the transcription start site (17). We now report that two sites that bind the transcription factor Sp1, located approximately 115 and 65 base pairs upstream from the transcription start site of the WAF1/CIP1 gene, are necessary for normal levels of basal, PMA-induced, and okadaic acid-induced transcription. These sites are also necessary for induction of the WAF1/CIP1 promoter by exogenous Sp1 in the Sp1-deficient Drosophila Schneider SL2 cell line. Finally, our observation that a reporter plasmid containing multiple Sp1 binding sites and a TATA box shows transcriptional induction in response to PMA and okadaic acid in U937 cells suggests that the activity of Sp1 is sufficient for induced transcription of the WAF1/CIP1 gene.

MATERIALS AND METHODS

Cell Culture and Conditions—U937 human myeloid leukemic cells obtained from ATCC (Rockville, MD) and Dr. D. Kirkways (East Carolina University, Greenville, NC) were passaged in Dulbeco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated bovine calf serum (Life Technologies, Inc.) and antibiotics at 37°C in 5% CO2. Drosophila Schneider SL2 cells were grown at room temperature in Schneider’s Drosophila medium (Life Technologies, Inc.) supplemented with 20% fetal calf serum.

RNA Isolation and Northern blot Analysis—Total cellular RNA was extracted by the guanidium thiocyanate-CsCl ultracentrifugation method. RNA was separated on 1.2% agarose gels containing formaldehyde and transferred to a membrane. To detect specific transcripts, 32P-cDNA probes labeled by random priming were hybridized to the membranes. The probes used were a 2.1-kb fragment containing an approximately full-length WAF1/CIP1 cDNA and a 1.5-kb tubulin cDNA.

Plasmids—The CAT reporter plasmid containing 2.3 kb of WAF1/CIP1 promoter sequence was a gift from T. Waldman and B. Vogelstein (Johns Hopkins University, Baltimore, MD). Smaller regions of the WAF1/CIP1 promoter were obtained by PCR amplification using the larger promoter construct as template and primers containing the desired 5’ and 3’ promoter sequences. Mutations introduced into 5’ primers were incorporated into the subsequent constructs. PCR products were first cloned into the pCRII vector (Invitrogen) and then moved into the CAT reporter plasmid pCAT1 modified by excision of the SV40 trimer cassette (18). The −122/−61 deletion construct was generated by excising sequences between Smal sites at −125 and −63 from...
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**FIG. 1. Accumulation of WAF1 mRNA in response to treatment of U937 cells with PMA or okadaic acid.** U937 cells were treated with 100 nM PMA, 100 nM okadaic acid, or 500 nM okadaic acid as indicated above the lanes. At the indicated times, cells were collected and used to prepare RNA for Northern blot analysis. 20 μg of total RNA was loaded in each lane, and filters were first probed with radiolabeled WAF1/CIP1 cDNA and then stripped and reprobed with tubulin cDNA as a loading control.

The construct containing promoter sequences from −154 to +16, 2.2 kb of upstream WAF1/CIP1 promoter sequence was inserted into this −122/−61 deletion construct using an Apal site at −129 and a vector HindIII site at the 5’ end of the promoter sequence. The −82/−62 deletion was made by cloning the −154/−16 fragment of promoter into the single-stranded vector M13mp18 and then using a primer containing sequences from either end of the desired deletion for site-directed mutagenesis. To construct the Sp1-dependent luciferase reporter plasmid pGAGC6, oligonucleotides containing consensus Sp1 binding sites were synthesized. A multimer of this oligonucleotide containing six Sp1 binding sites was inserted into the construct containing the Sp1 multimer at the 3’ end of the promoter sequence. The plasmid pGAM, containing only the adenovirus TATA box, was used as a control.

Transfection and Reporter Gene Assays—U937 cells were transfected by electroporation, and CAT assays were performed as described previously (19). 24 μg of WAF1/CIP1 promoter construct and 1 μg of cytomegalovirus-β-galactosidase plasmid as a transfection control were used per 10 million cells. After electroporation, cells were divided into three culture dishes and allowed to recover for 6 h in medium containing 25% fetal bovine serum. For experiments using antibodies, 1 μg of total cell protein was used to prepare RNA for Northern blot analysis. 20 μg of total RNA was loaded in each lane, and filters were first probed with radiolabeled cDNA and then stripped and reprobed with tubulin cDNA as a control.

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**FIG. 2. Promoter sequences between −122 and +16 mediate phorbol ester and okadaic acid induction of the WAF1/CIP1 promoter.** A, diagrammatic representation of CAT constructs containing between 2.3 kilobases and 170 bases of WAF1/CIP1 promoter sequence. All constructs shown have a 3’ terminator at +16, where +1 is the transcription start site. B, constructs were electroporated into U937 cells; one-third were treated with 200 nM PMA, one-third were treated with 100 nM okadaic acid, and one-third were untreated controls, as indicated below the panels. 24 h later CAT activity was assayed; for each construct, one assay is shown and average induction in response to PMA or OK calculated from three independent transfection experiments is listed below each lane. All transfections included cytomegalovirus-β-galactosidase plasmid; cell extracts were assayed for galactosidase activity to ensure equal transfection efficiency (galactosidase assays not shown). C, U937 cells were cotransfected with the indicated WAF1/CIP1 promoter constructs and vectors expressing either wild-type p53 (WT) or mutant p53 (Mut). CAT activity was assayed 24 h after transfection.

Gel Mobility Shift—For gel mobility shift experiments, oligonucleotides containing the desired promoter sequences were synthesized, annealed, and labeled using T4 polynucleotide kinase. After polyacrylamide gel purification, the labeled oligonucleotides were incubated with 5 μg of U937 nuclear extract in a buffer containing 70 mM KCl, 20 mM Hepes, pH 7.6, 1 mM dithiothreitol, 0.1 mM EDTA, 0.01 mM ZnCl₂, 60 μg/ml poly(dI-dC), and 30 μg/ml bovine serum albumin at 4°C for 30 min. Nuclear extracts were made as described (20) in a buffer containing 25% glycerol. For experiments using antibodies, 1 μl of anti-Sp1 polyclonal antiserum (21) or preimmune serum was added to the protein extract plus buffer, and a 15-min preincubation on ice was car-
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RESULTS

Treatment of U937 cells with either PMA or okadaic acid induces accumulation of WAF1/CIP1 mRNA (Fig. 1). Treatment with 100 nM PMA results in maximum levels of RNA by 2–4 h, while cells treated with 100 nM okadaic acid do not accumulate maximal levels of WAF1/CIP1 mRNA until 8 h (Fig. 1). This difference in the rate of induction between these agents is based on the concentration of activator employed, since higher concentrations of okadaic acid, 500 nM, result in increased levels of WAF1/CIP1 mRNA levels at earlier time points (Fig. 1).

To determine whether PMA and okadaic acid stimulate transcription from the WAF1/CIP1 promoter, constructs containing varying lengths of the WAF1/CIP1 promoter in front of a CAT reporter gene were transfected into U937 cells. Each set of transfected cells was split into equal aliquots, which were treated with PMA or okadaic acid or left untreated as controls. The smallest construct, containing 170 base pairs of promoter sequence from 154 bases upstream of the transcription start site at +1 to +16, was fully inducible by both PMA and okadaic acid (Fig. 2). When compared with the -2320/+16 construct (Fig. 2), the -154/+16 construct was more strongly induced by PMA (19.5-fold) and okadaic acid (16.1-fold), suggesting that upstream elements that repress transcription may be found between -2320 and -154.

A series of intermediate constructs displayed a gradual increase in response as promoter sequence was deleted from -2320 to -154, but all constructs were induced by PMA and okadaic acid. Two p53 binding sites identified at positions -2.3 kb and -1.4 kb do not play any role in the induction by these two agents since they are deleted in the smaller constructs without any affect on transcription. Further analysis of promoter sequences downstream from -154 revealed that a deletion of promoter sequences between -122 and -61 eliminated both basal and inducible promoter activity (see Fig. 3). When the -122/-61 deletion is introduced into a construct containing 2.3 kb of upstream WAF1/CIP1 promoter sequence, induction of transcription in response to PMA or okadaic acid is lost (Fig. 2), demonstrating that these sequences are important for inducible transcription and cannot be replaced by upstream elements. These elements are also important for induction in response to p53. Although U937 cells contain no wild-type p53, cotransfection of a wild type p53 expression vector induces transcription from the undeleted 2.3-kb WAF1/CIP1 promoter (Fig. 2). The -122/-61 deletion, however, abolishes induction in response to p53. Similar results were obtained when the two promoter constructs were compared in the GM glioma cell line, which contains a wild type p53 gene under the control of a steroid-inducible promoter (data not shown). These results indicate that WAF1/CIP1 promoter elements within the -122/-61 region are necessary for both p53-dependent and p53-independent induction.

The WAF1/CIP1 promoter sequence between -122 and -61 does not contain consensus binding sites for factors such as AP1, Egr-1, or NF-κB, which are known to activate other genes in response to phorbol esters or okadaic acid (22, 23). However, the region does contain several consensus binding sites for the transcription factor Sp1 (7). DNase I footprint analysis of this region indicated that at least two Sp1 consensus binding sites, centered around -115 and -67, fell within protected regions. PMA treatment of cells did not cause any noticeable change in the footprints, suggesting that the binding of factors to this region of the WAF1/CIP1 promoter is not enhanced by PMA. To verify that these potential Sp1 binding sites were important for induction of transcription in response to PMA and okadaic acid, a series of CAT constructs were generated with deletions or mutations of the Sp1 consensus sequences found in the -122/61 region.

2 J. Biggs, unpublished observations.
region of the WAF1/CIP1 promoter (diagrammed in Figs. 3 and 4). These constructs were transfected into U937 cells and analyzed for basal activity and for transcriptional response to PMA and okadaic acid (Figs. 3 and 4). As mentioned above, deletion of the 2122/261 sequence, which includes several Sp1 consensus binding sites, markedly decreased both basal and induced transcription. All transcription experiments included one transfection using the 2154/116 promoter construct; the basal transcription of this construct was set at 1.0, and basal transcription of all other constructs was normalized to this value. For each construct, percentage conversion of chloramphenicol to the acetylated form in PMA or okadaic acid–treated cells was divided by percentage conversion in untreated cells to obtain values for induced transcription.

Deletion of sequences between −131 and −117, immediately 5’ to the upstream Sp1 consensus binding site, decreased PMA induction by a small amount and decreased okadaic acid induction approximately 50%. A further deletion of sequences from −117 to −100, which eliminates the Sp1 binding sites entirely, markedly decreased basal transcription and induction in response to PMA and okadaic acid. In comparison, deletion of the downstream region between −81 and −62 knocked out okadaic acid response while having little effect on PMA response. PMA induction appears to require only the upstream element, while okadaic acid induction requires both the upstream and downstream elements.

To more precisely examine the role of the upstream Sp1 consensus site in mediating WAF1/CIP1 transcription, mutations were introduced into the 2131/116 promoter construct (Fig. 4). Mutation of three bases in the first Sp1 site decreased both basal and induced transcription by PMA and okadaic acid; the reduction in activity was approximately the same as that observed when the region was deleted. This result confirms that the Sp1 binding site is necessary for induction. Mutation of bases outside the Sp1 consensus sequence had little effect on promoter activity (Fig. 4).

To test for binding of Sp1 (or other factors) at these sites, double-stranded oligonucleotides containing WAF1/CIP1 promoter sequence from −128 to −99 or from −86 to −57 were used for gel mobility shift experiments with nuclear extracts from U937 cells (Fig. 5). Both oligonucleotides bind to a set of three proteins or protein complexes, which closely resemble the set of proteins previously observed to bind to both Sp1 sites and retinoblastoma control elements (24, 25). These proteins are usually designated 1A, 1B, and 2, (see Fig. 5); 1A has been identified as the Sp1 gene product based on interactions with
anti-Sp1 antibodies (25), and the other bands are postulated to be Sp1-related proteins (26). As shown in Fig. 5, preincubation of U937 nuclear extract with anti-Sp1 antibodies (21) disrupts interaction with Sp1-related proteins (26). As shown in Fig. 5, preincubation of U937 nuclear extract with anti-Sp1 antibodies (25) competes off the binding of a TBP-dependent complex. All three proteins (1A, 1B, and 2) can also be competed off the WAF1/CIP1 promoter oligonucleotide with excess unlabeled -128/-99 oligonucleotide or an oligonucleotide containing the SV 40 Sp1 binding site (Promega) but not by an oligonucleotide that does not contain an Sp1 consensus sequence (see “Materials and Methods” for sequence), suggesting that the proteins that bind to both footprinted regions are Sp1 or Sp1-related factors.

To verify that Sp1 activates transcription of the WAF1/CIP1 promoter, WAF1/CIP1 constructs were transfected into the Sp1-deficient Drosophila Schneider SL 2 cell line either in the presence or in the absence of the Sp1 expression vector pPSp1 (27). In the Sp1-deficient Drosophila cells, WAF1/CIP1 constructs containing sequence from -117 to +16 are highly induced in response to exogenous Sp1 expression (Fig. 6). Deletion of either the upstream or downstream Sp1 binding sites individually has a partial effect on the level of expression in response to Sp1, but deletion of both sites results in a much greater reduction in promoter activity induced by cotransfecting the Sp1 expression vector (Fig. 6). This result is similar to the pattern observed in U937 cells for response to PMA or okadaic acid, although deletion of individual sites can have a more severe effect on response to PMA or okadaic acid in the U937 cells.

Sp1 has been shown to interact with the TATA box binding protein (TBP)-associated protein TAF110 and, in collaboration with another TBP-associated protein, TAF250, activates transcription (28, 29). If Sp1 and the complex of TBP proteins are sufficient for induction of transcription in response to PMA or okadaic acid, it is predicted that transcription of a reporter plasmid containing multiple Sp1 binding sites and a TATA box would be stimulated by PMA or okadaic acid treatment of U937 cells. To evaluate this possibility the plasmid pGAGC6, containing six Sp1 binding sites and an adenovirus TATA box, was transfected into U937 cells, and the cells were treated with PMA or okadaic acid. Both PMA and okadaic acid induced transcription from this vector, while control vector not containing Sp1 sites was not induced (Fig. 7).

**Discussion**

The association of WAF1/CIP1 expression with differentiation in many types of cultured cells suggests that p53-independent induction of WAF1/CIP1 may have a role in cell differentiation in vivo; the pattern of WAF1/CIP1 expression during mouse embryogenesis also correlates with terminal differentiation of skeletal muscle, cartilage skin, and nasal epithelium (12). Identification of the WAF1/CIP1 promoter elements that mediate p53-independent induction of transcription should help to identify the signal transduction pathways that stimulate expression of WAF1/CIP1 during cell differentiation.

Since WAF1/CIP1 expression is stimulated by a number of agents, including platelet-derived growth factor, fibroblast growth factor, interleukin 2, tumor growth factor β, phorbol esters, okadaic acid, retinoic acid, and vitamin D₃ (15, 30), it is possible that a number of elements in the WAF1/CIP1 promoter function together to precisely regulate the level of WAF1/CIP1 expression. Such multiple signals might be necessary to generate sufficient WAF1/CIP1 expression for growth inhibition, since there is some evidence that the ratio of WAF1/CIP1 protein to target molecules, such as cyclin-dependent kinases, determines its effect (31). This idea is supported by the fact that the WAF1/CIP1 promoter element responsible for induction in response to serum in fibroblasts appears to be located between -1,817 and -4699 bases upstream of the transcription start site (14), while induction in response to tumor growth factor β in SW480 cells is mediated by elements between -611 base pair and -1.1 kb upstream (17). These results suggest that there are at least two p53-independent pathways for induction of WAF1/CIP1 transcription.

We now report that induction of the WAF1/CIP1 promoter in U937 leukemic cells by PMA and okadaic acid involves Sp1 binding sites located approximately 115 and 80 bases upstream of the transcription start site; loss of these sites results in lack of response to PMA and okadaic acid as well as loss of WAF1/CIP1 promoter induction in response to exogenous Sp1 in the Sp1-deficient Drosophila Schneider SL2 cell line. The upstream (-115) Sp1 site is vital for full response of the promoter to PMA in U937 cells, but all potential Sp1 binding sites between -122 and -61 must be deleted to abolish induction by exogenous Sp1 in the SL2 cells. The requirements for utilization of an Sp1 binding site in U937 cells may be more stringent due to the presence of Sp1-related proteins (26) or Sp1-inhibitory proteins (25) that are not present in the Drosophila SL2 cells. The fact that the reporter plasmid pGAGC6 (containing multiple Sp1 binding sites upstream of a TATA box) is induced by PMA and okadaic acid in U937 cells suggests that Sp1 may be sufficient for increased transcription of the WAF1/CIP1 promoter in response to PMA or okadaic acid treatment.

The Sp1 transcription factor is found in glycosylated and phosphorylated forms, but little is known about how these modifications affect function (32). Interactions between Sp1 and the retinoblastoma protein have also been reported; a 20-kDa inhibitor of Sp1 (Sp1-I) was identified that also bound to Rb, and it was proposed that Rb binds and inactivates Sp1-I, leading to transcriptional activation by Sp1 (24). A 74-kDa protein that binds to the transactivation domain of Sp1 and inhibits Sp1-mediated transactivation has also been identified (33). These reports suggest that there are multiple inhibitors of Sp1, which could inhibit interaction of Sp1 with the transcription factor IID complex (28). It is also possible that proteins of the transcription factor IID complex are modified in response to PMA, or that the composition of the transcription factor IID
complex is altered by gain or loss of TBP-associated factors (TAFs). Recent studies have shown that some TAFs may serve as coactivators to mediate transcriptional regulation (34).

Signal transduction pathways that activate or inactivate such Sp1 inhibitors may serve to regulate Sp1 transcriptional activation and may therefore regulate WAF1/CIP1 and other genes involved in cell differentiation. A number of signal transduction pathways composed of kinase cascades have been described in the literature (35). However, a variety of signals that activate either the mitogen-activated protein kinase pathway, the stress-activated protein kinase pathway, or the p38 kinase activation may be part of an as yet unidentified pathway. These signals include UV irradiation, activated mitogen-activated protein kinase pathway, and osmotic shock. This suggests that the signals that induce WAF1/CIP1 transcription via Sp1 may be part of an as yet unidentified pathway.

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