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Activation of the p21$^{Waf1/Cip1}$ Promoter by the *ets* Oncogene Family Transcription Factor E1AF

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p21$^{Waf1/Cip1}$ is one of the key regulatory proteins in cell cycle, terminal differentiation, and apoptosis. Its promoter was shown to be transactivated by the wild-type p53 protein as well as in a p53-independent manner. In this report, we demonstrate that E1AF, an *ets*-related transcription factor, activates the human p21$^{Waf1/Cip1}$ promoter by interacting with the *ets*-binding sites located close to the two previously identified p53-responsive elements. Northern blot analysis revealed that p21$^{Waf1/Cip1}$ and E1AF were correlatively upregulated in response to cisplatin treatment in SiHa cells. Transient expression assays demonstrated that E1AF can activate the p21$^{Waf1/Cip1}$ promoter-driven luciferase reporter gene in SiHa cells. The p21$^{Waf1/Cip1}$ promoter activity was also increased in p53-null Saos2 osteosarcoma cells, but was markedly reduced when the *ets*-binding sites were deleted. These results indicate that E1AF positively regulates transcription from the p21$^{Waf1/Cip1}$ promoter in response to genotoxic stresses.

The p21$^{Waf1/Cip1}$ gene is located on chromosome 6p21.2 and its protein product forms a quaternary complex with cyclin, cyclin-dependent kinase (cdk) and the proliferating cell nuclear antigen (PCNA) (1-4). p21$^{Waf1/Cip1}$ was shown to inhibit the kinase activity of cdks and to play a key role in cell-cycle arrest (5, 6). It was also reported that the p21$^{Waf1/Cip1}$ protein inhibits DNA replication by interacting with the proliferating cell nuclear antigen (PCNA) but does not inhibit the DNA-repairing activity of PCNA (7, 8). Previous reports indicated that p21$^{Waf1/Cip1}$ was overexpressed when cells were under the following conditions: senescence, serum deprivation, contact inhibition, differentiation and exposure to several genotoxic stresses (4, 9-12). Thus, p21$^{Waf1/Cip1}$ acts as a critical regulator of growth control.

The p21$^{Waf1/Cip1}$ promoter was shown to be transactivated by wild-type p53, a tumor suppressor gene (2,10) thought to bind to the p53-responsive elements located 1.3 and 2.2 kb upstream of the first exon of the human p21$^{Waf1/Cip1}$ gene (13). However, some reports indicated that p21$^{Waf1/Cip1}$ is expressed in cells that lack p53 or possess a mutant p53 gene, suggesting that p53 is not the only transactivator of the p21$^{Waf1/Cip1}$ promoter (14-18). Macleod *et al.* (12) reported that in the mouse p21$^{Waf1/Cip1}$ promoter region a potent *ets*-binding sequence located at position −2,237 overlaps the p53-binding site, but the transcriptional significance of this overlap remains to be elucidated.

E1AF, an *ets*-oncogene family transcription factor, was isolated by its ability to bind to the enhancer element of the human adenovirus E1A gene (19, 20). Our previous study showed that E1AF positively regulates the expression of matrix metalloproteinase genes associated with cancer cell invasion (21- 23).

Here we report that the expression of E1AF mRNA was upregulated by treatment with the anti-cancer drug cisplatin and that this increase correlated with the increase in p21$^{Waf1/Cip1}$ expression. Furthermore, we show that in transient transfection assays E1AF can activate the p21$^{Waf1/Cip1}$ promoter in p53-null cells and in cells in which p53 is degraded by the HPV E6 protein. The promoter activity of p21$^{Waf1/Cip1}$ was decreased when the *ets*-binding sequence located close to the p53-binding sites was deleted. These observations indicate that E1AF stimulates transcription from the p21$^{Waf1/Cip1}$ promoter when cells are exposed to genotoxic stresses.

MATERIALS AND METHODS

Cell culture and treatment with cisplatin. HPV 16-positive SiHa cell line was used. Cells were maintained at 37°C and 5%

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CO_2 in Dulbecco's modified Eagles medium (DMEM) supplemented with 8% fetal bovine serum (Filtron Pty Ltd, Brooklin, Australia) and penicillin/streptomycin/fungizon (Gibco BRL, Gaithersburg, MD, USA). After cells reached 80% confluency in normal growth medium, 5 μg/ml, 10 μg/ml or 20 μg/ml of cisplatin were added to the medium for 24 h.

RNA isolation and Northern blotting. Cells were suspended in a hypotonic buffer and lysed with NP-40 as previously described (24). Cytoplasmic RNA was extracted twice with a phenol/chloroform/isoamylalcohol (25:24:1) and precipitated with two volumes of ethanol. Twenty μg of total cell RNA was denatured at 94°C for 5 min in the loading buffer composed of 17.5% formaldehyde, 50% formamide and 1× MOPS, and subjected to electrophoresis in a 1.2% agarose gel containing 2.2M formaldehyde and 1× MOPS. After electrophoresis, RNA was transferred onto a nitrocellulose membrane (Schleichner and Schuell, Dassel, Germany) by capillary blotting. The membrane was dried and baked at 80°C for 2 hours. Prehybridization was carried out at 42°C in 5× SSPE, 40% formamide, 5× Denhardt's solution and 0.1 mg/ml denatured herring sperm DNA for 2 hours. Hybridization was performed at 42°C for 24 hours in the presence of either [^{32}P]-labeled E1AF or p21$^{Waf1/Cip1}$ probes that were labeled with a BcaBest Labeling Kit (TAKARA, Tokyo, Japan). The filters were washed twice with 1×SSC/0.1% SDS at room temperature for 20 min, twice with 0.2×SSC/0.1% SDS at 55°C for 30 min, and then exposed to Kodak X-O Mat X-ray films at −80°C for 48 hours.

Transient expression assays. SiHa and Saos2 cells were used for these assays. Cells were grown to 50% confluency in 60-mm tissue culture plates and cotransfected with a luciferase reporter plasmid and either the E1AF expression vector pCMVE1AF (2.7 μg, 5.4 μg) (21,23). or the parental vector pEV3S (25) plus one of the reporter plasmids: WWP-Luc, DM-Luc (2) or DMPst-Luc (2 μg) by lipofection method using Lipofectamine (Gibco BRL Gaithersburg, MD, USA). Forty-eight hours post transfection, cells were harvested and luciferase activity was measured.

Transfected cells were lysed with a lysis buffer and analyzed with the luciferase assay system (Promega, Madison, WI, USA). Light emission was measured with a scintillation counter. The quantity of protein was normalized to the number of cells. 2×10^6 SiHa cells and 1×10^6 Saos2 cells were analyzed in each experiment.

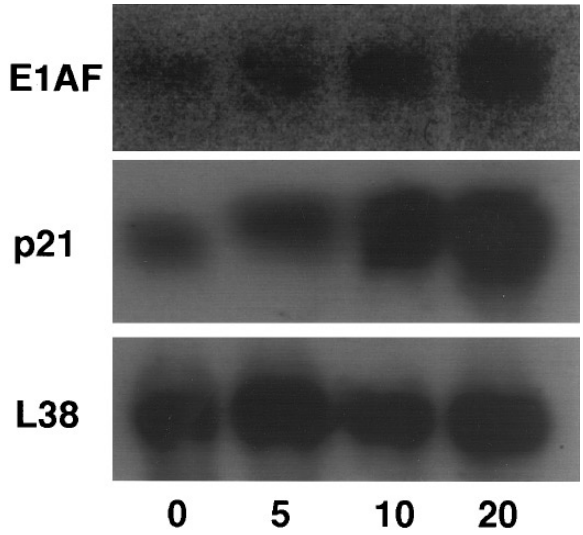

FIG. 1. Northern blot hybridization of E1AF and p21$^{Waf1/Cip1}$ in cisplatin-treated SiHa cells. The E1AF mRNA was increased in a cisplatin dose-dependent manner (the number indicates the amount of cisplatin in microgram/ml) which is correlated with the expression of p21$^{Waf1/Cip1}$. The membrane was rehybridized with ^{32}P-labeled L38, a ribosomal subunit specific probe.

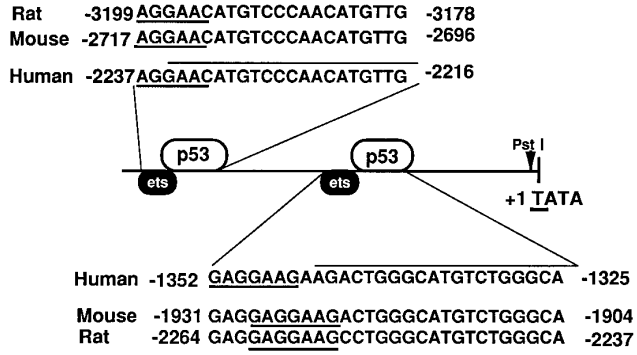

FIG. 2. The *ets*- and p53-binding motifs located in the promoter region of human p21$^{Waf1/Cip1}$. The *ets*-binding sites (5′-GGAA/T-3′) near the p53-responsive elements are shown. The 2.2kb upstream p53- and *ets*-binding sites were deleted in the DM-Luc reporter plasmid. The DMPst-Luc plasmid which contains only a TATA box was obtained by digesting WWP-Luc with Pst I and religation. Relevant mouse and rat sequences are also shown with relative position numbers.

RESULTS

SiHa cells harboring human papillomavirus were treated with increasing amounts of cisplatin and total cell RNA was isolated. The steady-state levels of E1AF and p21$^{Waf1/Cip1}$ mRNA were analyzed by Northern blot hybridization. Increased steady-state levels of E1AF mRNA were observed in cells treated with cisplatin in a dose-dependent manner. p21$^{Waf1/Cip1}$ mRNA was upregulated in response to cisplatin treatment and correlated with E1AF expression (Fig. 1). Filters were then rehybridized with a ^{32}P-labeled L38, a ribosomal subunit probe to confirm equal RNA loading.

We compared *ets*-binding motifs of human, mouse and rat p21$^{Waf1/Cip1}$ promoter sequences as shown in Figure 2. The *ets*-binding motif (5′-GGAA/T-3′) is located near the upstream and downstream p53-responsive elements. These two regions are highly conserved in the mouse and rat p21$^{Waf1/Cip1}$ promoters. We analyzed the role of these two *ets*-binding motifs on the p21$^{Waf1/Cip1}$ promoter activity in transient transfection assays. A luciferase reporter gene driven by the human p21$^{Waf1/Cip1}$ promoter (WWP-Luc) was cotransfected with increasing amounts of the E1AF-expressing plasmid (pCMVE1AF) into SiHa cells by the lipofection method. Forty-eight hours post transfection, cells were lysed and the emission of light by luciferase was measured using a scintillation counter. More than a 40-fold increase of luciferase activity was observed in cells cotransfected with 5.4 μg of pCMVE1AF as compared to luciferase activity in cells cotransfected with the parental vector pEV3S (Fig. 3a). Next, the DM-Luc and DMPst-Luc reporter plasmids were cotransfected with

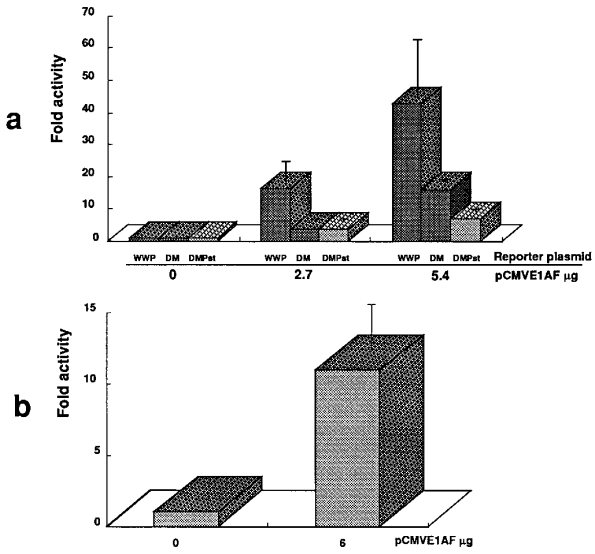

FIG. 3. E1AF activates the human p21$^{Waf1/Cip1}$ promoter. Average and standard errors of fold activities for each concentration of pCMVE1AF are indicated. (a) More than a 40-fold increase in light emission was observed in cotransfections with 5.4 μg of pCMVE1AF compared to the result obtained with the parental vector. The activity was reduced when cells were cotransfected with the DM-Luc reporter plasmid as compared to that in cells transfected with WWP-Luc. Significant reduction of light emission was observed at each concentration of the expression vector. (b) E1AF activates the p21$^{Waf1/Cip1}$ promoter in a p53-independent manner. In p53-null Saos2 osteosarcoma cells cotransfected with E1AF and the reporter plasmid, more than a 10-fold increase in luciferase activity was observed.

pCMVE1AF for transient promoter assays. DMLuc is a reporter plasmid that lacks the upstream p53- and *ets*-binding motifs but contains the downstream sites. The DMPst-Luc reporter plasmid contains only a TATA box, but not the p53 or *ets*-binding motifs. The luciferase activity was reduced less than half in cells transfected with the DM-Luc or DMPst-Luc reporter plasmids compared to the activity measured in cells transfected with the intact WWP-Luc plasmid (Fig. 3a).

We then performed the same type of analysis in p53-deficient Saos2 cells. As shown in Figure 3b, luciferase activity in cells cotransfected with the reporter plasmid and pCMVE1AF was more than 10-fold higher than in cells transfected with the reporter plasmid alone.

DISCUSSION

Cisplatin is a commonly used chemotherapeutic agent against cancer. It cross-links DNA and induces DNA strand breaks (26), and was reported to induce p21$^{Waf1/Cip1}$. El-Deiry *et al.* (13) defined the two p53-responsive elements in the p21$^{Waf1/Cip1}$ promoter that are present in the mouse and rat nucleotide sequences and conserved in their positions relative to the transcription initiation site. p21$^{Waf1/Cip1}$ was initially reported to be transactivated by the wild-type p53 protein. Recent reports, however, demonstrated that p21$^{Waf1/Cip1}$ is upregulated also by a p53-independent pathway. Michieli *et al.* (14) reported that p21$^{Waf1/Cip1}$ was induced in embryonic fibroblasts isolated from p53 knock-out mice as well as in normal cells treated with certain growth factors, but was not expressed in p53-deficient cells exposed to DNA damage elicited by gamma-irradiation. The authors suggested the existence of two separate pathways for the induction of p21$^{Waf1/Cip1}$: a p53-dependent pathway activated by DNA damage and a p53-independent one activated by mitogens at the entry into the cell cycle. In addition, Gartenhaus and colleagues (27) reported that p21$^{Waf1/Cip1}$ mRNA and protein were rapidly induced in response to treatment with Adriamycin, a genotoxic agent, regardless of the p53 status of the cell lines. They also demonstrated that apoptosis was induced in these cell lines despite some having a functionally inactive p53 protein. Jiang *et al.* (28) showed that expression of a specific melanoma differentiation-associated (mda) cDNA, mda-6, which is identical to p21$^{Waf1/Cip1/Sdi1}$, is inversely correlated with melanoma progression and growth. These results suggest that the p53 independent expression of p21$^{Waf1/Cip1}$ could occur in cellular differentiation, growth control and apoptosis.

In the present study, we investigated E1AF and p21$^{Waf1/Cip1}$ mRNA expression in response to cisplatin treatment. Both E1AF and p21$^{Waf1/Cip1}$ were correlatively upregulated by genotoxic stresses induced by cisplatin treatment in SiHa cells harboring HPV 16. Ets proteins have been implicated in the regulation of gene expression during several biological processes including growth control, cellular differentiation and control of tumorigenicity (29). Macleod *et al.* (12) reported that in the mouse p21$^{Waf1/Cip1}$ promoter region the *ets*-binding site overlaps the p53-responsive element located at position −1941 relative to the transcription initiation site. Here we identified *ets*-binding sites near the p53-responsive elements in the upstream promoter region of human p21$^{Waf1/Cip1}$ (the sequence has already been determined by el-Deiry *et al.*, 1995), which were highly conserved in mouse and rat. Moreover, we showed that E1AF can activate the promoter activity of p21$^{Waf1/Cip1}$ in transient transfection assays in SiHa cells. Although endogenous p53 protein in SiHa cells was originally thought to be degraded by the human papillomavirus E6 protein, Butz *et al.* reported the existence of a functional p53 protein in these cells (30). To exclude the effects of the residual endogenous p53 protein on the p21$^{Waf1/Cip1}$ promoter, we performed a transient expression assay in a p53-null Saos2 osteosarcoma cell line (31). E1AF activated the p21$^{Waf1/Cip1}$ promoter in Saos2 cells in a p53-independent manner. The luciferase activity in cells transiently transfected with the *ets* deletion mutants (DM-Luc or DMPst-Luc) was markedly reduced, indicating that the *ets*-binding site positioned

2.2 kb upstream of the TATA box plays a critical role in the E1AF-mediated transactivation of $p21^{Waf1/Cip1}$. However, it remains to be determined which regions of the other *ets*-binding motifs contribute to the residual activity of the promoter.

The malignant phenotype has been defined as an uncontrolled growth and/or metastasis (invasiveness) of tumor cells. Suzuki *et al.* reported that overexpression of wild-type ETS1 induces reduction of tumorigenicity in human colon cancer cells (32). It was also reported that the ETS2 protein can activate expression of the p53 tumor suppressor gene (33). These data suggest that expression of Ets proteins strongly correlates with suppression of tumorigenicity. Our results suggest that E1AF may function as a growth suppressor by activating the expression of $p21^{Waf1/Cip1}$.

However, it was demonstrated that E1AF can activate matrix metalloproteinase (MMP) gene promoters and confer invasive phenotype on human cancer cells (21-23). These results suggest that E1AF may play an antagonistic function on malignant behavior of tumor cells: it can be a positive regulator of invasion by transactivating MMP genes and a negative regulator of growth by transactivating the $p21^{Waf1/Cip1}$ gene. In summary, we conclude that E1AF, an Ets-related protein, function as a transactivator of the human $p21^{Waf1/Cip1}$ gene and in this way participate in growth control.

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