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Activation of the Human p27^{Kip1} Promoter by IFNα 2b

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p27^{Kip1} is one of the key regulatory proteins in cell cycle through inhibition of pRB phosphorylation by suppression of the activity of several cyclin/Cdk complexes. The expression of p27^{Kip1} has been shown to be controlled by a posttranslational mechanism, although vitamin D_3 and neuronal differentiation can also induce its mRNA. Recently, the p27^{Kip1} promoter was isolated and sequenced from a human leukocyte genomic library. In this report, we demonstrate that IFNα 2b, activates the human p27^{Kip1} promoter-driven luciferase reporter gene in transient expression assays in H82 cells. This induction might involve two IRF 1-like binding sites present in the p27^{Kip1} promoter. To our knowledge this is the first report on the direct activation of the human p27^{Kip1} promoter by IFNα 2b.

Key Words: **interferon α 2b; p27^{Kip1}; promoter; luciferase; H82/IRF-1.**

Mitogen-dependent progression through the G1 and S phases of the eukaryotic cell cycle are cooperatively regulated by several classes of cyclin-dependent kinases (CDKs) whose activities are in turn constrained by CDK inhibitors (CKIs) (1). CKIs that govern these events have been assigned to one of two families based on their structure and CDK targets (2). One group, comprised of related proteins known as the Cip/Kip family, p21$^{Waf1/Cip1}$, p27^{Kip1} and p57^{Kip2}, appears to function as broad specificity inhibitors (3–5). These inhibitors have a conserved amino-terminal 60-residue domain responsible for kinase binding and inhibition (6).The second class of CKIs, referred to as the INK4 (Inhibitor of CDK4) family, is comprised of ankyrin repeat proteins and includes the p15^{Ink4b}, p16^{Ink4a}, p18^{ink4c}, and p19^{Ink4d} proteins and share no homology with the Cip/Kip family (7–10). The p27^{Kip1} gene is located on chromosome 12p13 and its protein product inhibits cell growth by binding to CDKs, although, recently, a role for both p27^{Kip1} and p21^{Waf1} as positive regulators of cyclin D-CDK complexes has been demonstrated (11, 12). p27^{Kip1} was discovered as an inhibitory activity induced by antimitogenic signals (13), its expression increases in cells grown to high density or in those deprived of serum mitogenics (14). Antimitogenic agents such as cyclic AMP, rapamycin and Interferon β also stimulate expression of p27 (15–17). Unlike other mammalian cell cycle proteins, for which correlation have been found between the abundance of these proteins and changes in the amount of mRNA present (18), the variations in the amount of p27^{Kip1} occur in the presence of constant amounts of mRNA (19, 20). However, recent reports showed that p27^{Kip1} mRNA is induced by vitamin D3 in U937 cells (21), and by neuronal differentiation (22), suggesting that transcriptional regulation of the p27^{Kip1} gene might be also important in controlling cell cycle. Likewise, the human p27^{Kip1} promoter was recently cloned and characterized (23). Deletion analysis suggested that a region from −774 to −435 relative to the initiating codon could contain the essential transcriptional factor binding sites. Interferons (IFNs) are important cytokines with pleiotropic effects on different cells, ranging from antiviral and antiproliferative effects to augmentation of the immune response (24). These effects involve transcriptional and/or translational regulation of a set of effector genes along the IFN-signaling pathway leading to control of cell growth and differentiation; host defense against viral infections and triggering of a series of defense mechanism of the immune system (25). The growth inhibitory actions of IFNs are thought to be mediated through the induction of IFN-inducible gene products. Among them is a growing family of transcription factors, the Interferon Regulatory Factors (IRFs). IRF-1 and IRF-2 are the best characterized members of this family (26), IRF-1 activates transcription, whereas IRF-2 inhibits it (27). Computer analysis of the p27^{Kip1} promoter sequence by the SSCAN Software (28) revealed that there are two putative Interferon Response Elements (IRE) at the bases −483 and −462 of the minus strand. We have previously shown in kinetic experiments that IFNα 2b induced the p27^{Kip1} mRNA at 48 h in H82 cells (29). Here, we report that in transient transfection, IFNα 2b can ac-

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FIG. 1. A fragment of the human p27^{Kip1} promoter containing two tandemly repeated IRF-1 sites shown in the open boxes. IRF-1 consensus motifs are indicated below the promoter sequence in capital bold letters.

tivate the p27^{Kip1} promoter in H82 cells using a luciferase reporter vector. This observation indicates that IFNα 2b stimulates transcription from the p27^{Kip1} promoter when cells are exposed to this cytokine most probably through induction of IRF-1 and that this induction might lead either to inhibition of cell growth or apoptosis.

MATERIALS AND METHODS

Cell culture. H82, a small cell lung carcinoma (SCLC) cell line, was maintained at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco-BRL) and penicillin/streptomycin. Human recombinant IFNα 2b (specific activity, 5×10^7 IU/mg protein) was obtained from Heber Biotec, Cuba. For the kinetic experiment H82 cells were cultured in the presence and absence of IFNα 2b (500 IU/ml) for up to 48 h. Cell extracts were prepared at different time points after IFNα 2b addition.

Promoter amplification by PCR. Genomic DNA was isolated from H82 cells with the Wizard Genomic DNA Purification System (Promega). DNA concentration was determined spectrophotometrically from absorbance at 260 nm. Total DNA was characterized by ethidium bromide staining of agarose gels to ensure the use of only intact DNA. Intact total DNA (1 μg) was used as template to amplify an 800 bp fragment of the p27^{Kip1} promoter corresponding to the region -800 to -1 relative to the initiating codon by using polymerase chain reaction (PCR). The PCR primers were synthesized from the p27^{Kip1} promoter sequence reported elsewhere (24). The sequences of primers are as follows: 5′-ACTAAGCTTCGAACCCGCCAACGCAGCGCC-3′ and 5′-AGTAAGCTTCTTTCTCCCGGGTCTGCACGA-3′.

The PCR reaction mixture contained 1 μg genomic DNA template, 10 mM Tris-HCl (pH 8.4); 50 mM KCl; 2.5 mM $MgCl_2$; 0.2 mM each dATP, dCTP, dGTP, and dTTP; 0.5 μM 5′-primer, 0.5 μM 3′-primer and 5 U *Taq* DNA Polymerase (ENZIBIOT, Cuba). PCR was performed with a DNA Thermal Cycler (M.J. Research). Denaturation was at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min for 30 cycles. The amplified 800 bp DNA fragment containing human p27^{Kip1} promoter was sub-cloned into the pMOS*Blue*-T vector (Amersham), fully sequenced, digested with the *Hin*dIII restriction enzyme and further cloned into the *Hin*dIII site of the pXP1 promoter-less plasmid containing the luciferase reporter gene.

Western blot analysis. H82 cells (4×10^6) were lysed in 20 mM Hepes, pH 7.4; 100 mM NaCl; 100 mM NaF; 1 mM sodium vanadate; 5 mM EDTA; 0.3% Triton X-100; 25 μg/ml protease inhibitor cocktail (leupeptin, aprotinin, pepstatin); 1 mM PMSF. Cell extracts (30 μg) were separated on a 10% acrylamide gel and electrotransferred onto nitrocellulose membrane (Amersham) and incubated with an anti-IRF-1 (sc 497, Santa Cruz) at a final concentration of 2 μg/ml. The proteins bands were detected using a peroxidase conjugate anti-rabbit (Amersham) and ECL detection kit (Amersham).

Transient expression assays. H82 cells were seeded (1×10^6) in 60-mm tissue culture plates and transfected either with the pXP27 (p27-promoter-luciferase reporter plasmid) (5 μg) or the pXP1 (parental vector) (5 μg) or the pGL2 (control vector) (Promega) (5 μg) by lipofection using Lipofectamine (Gibco-BRL). Twelve hours post transfection, recombinant human IFNα 2b was added at a concentration of 500 IU/ml. Forty-eight hours after IFNα 2b addition, cells were harvested and luciferase activity measured. Transfected cells were lysed and analyzed with the luciferase assay system (Promega). Light emission was measured in a MiniLumat LB 9506 (EG & G Berthold). Variations in transfection efficiency were accounted for by normalizing to a co-transfected cytomegalovirus promoter-driven-β-galactosidase control plasmid (0.25 μg). β-Galactosidase activity was measured in an ELISA-Photometer, Titertek Multiscan Plus. Data are presented as relative light units (RLU). Results shown are the means of duplicate assays.

RESULTS AND DISCUSSION

IFNs have been described as cytokines with cell growth inhibitory capacities. This antiproliferative action has been proposed to be of a major importance in the antitumor response. The exact mechanisms behind the ability of IFNs to arrest cell growth are still unknown. One hypothesis is that IFNs could induce the expression of the CDK inhibitors, which in turn would block the action of cyclin-CDKs complexes, bringing as a consequence that the retinoblastoma protein (pRB) remains unphosphorylated and active.

In the present study, we examined the effect of IFNα 2b on the p27^{Kip1} promoter activity in transient transfection assays. We constructed a reporter plasmid, pXP27 which contains an 800 bp fragment of the human p27^{Kip1} promoter corresponding to the region -800 to -1 relative to the initiating codon by using polymerase chain reaction (PCR) driving transcription of the luciferase reporter gene. This fragment was fully sequenced (data not shown). Interestingly, we found no nucleotide differences in this 800 bp fragment compared to the previously published p27^{Kip1} promoter sequence, which was isolated from a different source, a human leukocyte genomic library (23). Theoretically, this conserved nucleotide sequence between two different types of cells could mean that the p27^{Kip1} promoter is equally regulated in different cells types but must also consider the availability of cell specific transcriptional factors, which generates a tissue-specific differential transcriptional regulation. This would explain induction of the p27^{Kip1} mRNA in gastric carcinoma cell lines upon IFNβ addition (17) while in an independent set of experiments done in hematopoietic cell lines, IFNα (also an IFN type I) did not affect p27^{Kip1} mRNA levels (30).

It has been shown that IRF-1 activates a set of chemically synthesized, multimerized 6 bp sequences that

contains IRF binding sites, $AA^{A}/_{G}{}^{G}/_{T}GA$ (31). This region is responsible for the induced transcriptional activation of IFNβ gene (32). Each of these units cooperates for the maximal induction of the gene. Within the human $p27^{Kip1}$ promoter (23), we found two adjacent potential IRF binding motifs at the base −483 and −462 of the minus strand (Figure 1). To investigate the effect of IFNα 2b on the $p27^{Kip1}$ promoter activity in transient transfection assays, a luciferase reporter gene driven by the human $p27^{Kip1}$ promoter (pXP27) was transfected into H82 cells by lipofection. Forty-eight hours after IFNα 2b addition cells were lysed and the emission of light by luciferase was measured. More than 2-fold increase was observed in cells transfected with the pXP27 plasmid and treated with IFNα 2b for 48 h as compared to luciferase activity in untreated cells also transfected with the pXP27 (Figure 2). This activation of the $p27^{Kip1}$ promoter obtained in transient transfection experiments agrees with the previously observed increase in the $p27^{Kip1}$ mRNA in IFNα 2b-treated H82 cells (29). Furthermore, we have examined the induction of the IRF-1 transcription factor in these cells. The data shown in Figure 3 indicate that IFNα 2b is able to induce a peak of IRF-1 at 8 h as revealed by Western blot. This induction supports the role of IRF-1 as mediator of the IFNα 2b-induced $p27^{Kip1}$ activation through the two IRF-1 binding motifs. Additional studies are needed to show that these two sites are indeed important in the transcriptional activation of the intact $p27^{Kip1}$ promoter. Since the initial paper describing the p27 inhibitory activity (4), several mechanisms of $p27^{Kip1}$ regulation either translational or post-translational have been proposed (33–38). Therefore, this is the first report showing a direct effect of IFNα 2b on the transcriptional activation of the $p27^{Kip1}$ promoter. One fact is that $p27^{Kip1}$ regulation seems to be dependent of cell context. The activation of $p27^{Kip1}$ by IRF-1 may differ with the cell type, presumably due to the presence or absence of cell-type specific factors, which may direct IRF-1 to a particular set of promoters. The malignant phenotype has been defined as an uncontrolled growth of tumor cells. To identify new target genes of IRF-1 which are critical in the induction of these IRF-1-mediated cellular responses may be very useful to gain further insights into the cellular mechanisms of tumor suppression.

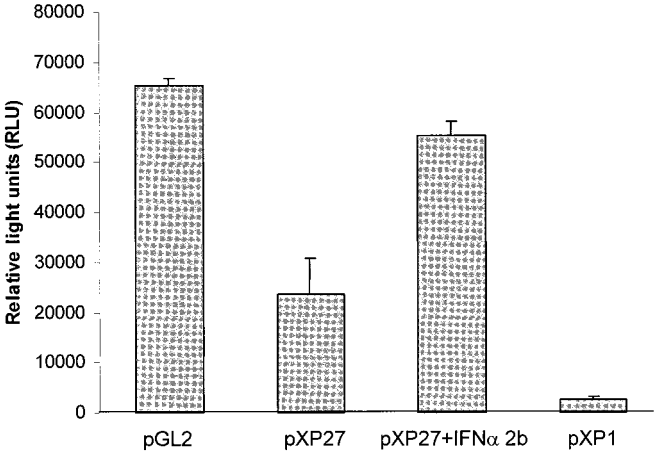

FIG. 2. Activation of the human $p27^{Kip1}$ promoter by IFNα 2b. H82 cells were transfected with pXP27-Luc (5 μg) and treated or not with IFNα 2b for 48 h. pGL2 (5 μg) and pXP1 (5 μg) were used as positive and negative controls respectively. Data are expressed as normalized luciferase values according to β-galactosidase activity.

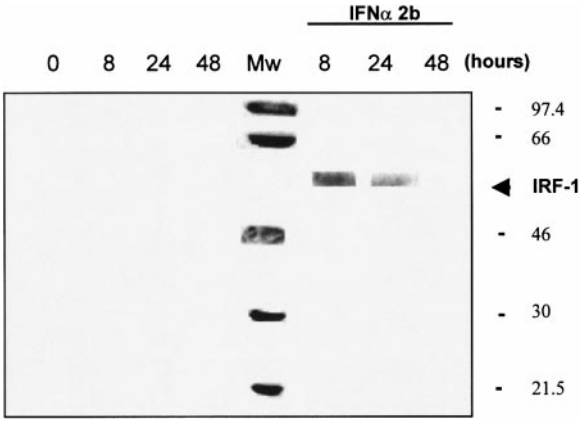

FIG. 3. IRF-1 protein level at various time points in H82 cells incubated or not with 500 IU/ml of human IFNα 2b for 48 h. IRF-1 expression was determined by Western blot. Arrowhead indicates the IFNα 2b-induced IRF-1. High Range Rainbow protein molecular weight markers (Amersham) were used.

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REFERENCES

1. Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* **13,** 1501–1512.
2. Sherr, C. J., and Roberts, J. M. (1995) *Genes Dev.* **9,**1149–1163.
3. El-Deiry, W. S., Tokino, T., Velcuscu, V. E., Levy, D. E., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Cell* **75,** 817–825.
4. Polyak, K., Lee, M. H., Erdjument Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massagué, J. (1994) *Cell* **78,** 59–66.
5. Lee, M. H., Reynisdóttir, I., and Massagué, J. (1995) *Genes Dev.* **9,** 639–649.
6. Harper, J. W., Elledge, S. J., Keyomarski, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowlski, S., Bai, C., Connell-Crowley, L., Swindell, E., Fox, M. P., and Wei, N. (1995) *Mol. Biol. Cell* **6,** 387–400.
7. Hannon, G. J., and Beach, D. (1994) *Nature* **371,** 257–260.
8. Serrano, M., Hannon, G. J., and Beach, D. (1993) *Nature* **366,** 704–707.
9. Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994) *Genes Dev.* **8,** 2939–2952.

10. Chan, F. K. M., Zhang, J., Chen, L., Shapiro, D. N., and Winoto, A. (1995) *Mol. Cell. Biol.* **15,** 2682–2688.
11. LaBaer, J., Garret, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. (1997) *Genes Dev.* **11,** 847–862.
12. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M., and Sherr, C. J. (1999) *EMBO J.* **18,** 1572–1583.
13. Polyak, K., Lee, M. H., Erdjument Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massagué, J. (1994) *Cell* **78,** 59–66.
14. Coats, S., Flanagan, W. M., Nourse, J., and Roberts, J. M. (1996) *Science* **272,** 877–880.
15. Kato, J., Matsuoka, M., Polyak, K., Massagué, J., and Sherr, C. J. (1994) *Cell* **79,** 487–496.
16. Nourse, J., Firpo, E., Flanagan, M., Meyerson, M., Polyak, K., Lee, M. H., Massagué, J., Crabtree, G., and Roberts, J. M. (1994) *Nature* **372,** 570–573.
17. Kuniyasu, H., Yasui, W., Kitahara, K., Naka, K., Yokozaki, H., Akama, Y., Hamamoto, T., Tahara, H., and Tahara, E. (1997) *Cell Growth Differ.* **8,** 47–52.
18. Murray, A., and Hunt, T. (1993) The Cell Cycle: An Introduction. Freeman, New York.
19. Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995) *Science* **267,** 1018–1021.
20. Hengst, L., and Reed, S. I. (1996) *Science* **271,** 1861–1864.
21. Liu, M., Lee, M. H., Cohen, M., Bommakanti, M., and Freedman, L. P. (1996) *Genes Dev.* **10,** 142–153.
22. Poluha, W., Poluha, D. K., Chang, B., Crosbie, N. E., Schonhoff, C. M., Kilpatrick, D. L., and Ross, A. H. (1996) *Mol. Cell. Biol.* **16,** 1335–1341.
23. Minami, S., Ohtani-Fujita, N., Igata, E., Tamaki, T., and Sakai, T. (1997) *FEBS Lett.* **411,** 1–6.
24. Clemens, M. J., and McNurlan, M. A. (1985) *Biochem J.* **226,** 345–360.
25. Baron, S., Tyring, S. K., Fleischmann, W. R., Jr., Coppenhaver, D. H., Niesel, D. W., Klimpel, G. R., Stanton, G. J., and Hughes, T. K. (1991) *J. Am. Med. Assoc.* **266,** 1375–1383.
26. Fujita, T., Sakakibara, J., Sudo, Y., Miyamoto, M., Kimura, Y., and Taniguchi, T. (1988) *EMBO J.* **7,** 3397–3405.
27. Harada, H., Fujita, T., Miyamoto, T., Kimura, Y., Maruyama, M., Furia, A., Miyata, T., and Taniguchi, T. (1989) *Cell* **58,** 729–739.
28. Prestridge, D. S. (1991) *CABIOS* **7,** 203–206.
29. Moro, A., Calixto, A., Suárez, E., Araña, M. J., and Perea, S. E. (1998) *Biochem. Biophys. Res. Commun.* **245,** 752–756.
30. Sangfelt, O., Erickson, S., Castro, J., Heiden, T., Gustafsson, A., Einhorn, S., and Grander, D. (1999) *Oncogene* **18,** 2798–2810.
31. Fujita, T., Shibuya, H., Hotta, H., Yamanishi, K., and Taniguchi, T. (1987) *Cell* **49,** 357–367.
32. Ohno, S., and Taniguchi, T. (1983) *Nucleic Acids Res.* **11,** 5403–5412.
33. Mandal, M., Bandyopadhyay, D., Goepfert, T. M., and Kumar, R. (1998) *Oncogene* **16,** 217–225.
34. Vlach, J., Hennecke, S., and Amati, B. (1997) *EMBO J.* **16,** 5334–5443.
35. Tomoda, K., Kubota, Y., Kato, J. (1999) *Nature* **398,** 160–165.
36. Shirane, M., Harumiya, Y., Ishida, N., Hirai, A., Miyamoto, C., Hatakeyama, S., Nakavama, K., and Kitagawa, M. (1999) *J. Biol. Chem.* **274,** 13886–13893.
37. Nakayama, K., and Kitagawa, M. (1999) *J. Biol. Chem.* **274,** 13886–13893.
38. Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. *Genes and Dev.* **13,** 1181–1189.