Modulation of E2F Activity Is Linked to Interferon-induced Growth Suppression of Hematopoietic Cells*

Satsuki Iwase‡, Yusuke Furukawa‡, Jiro Kikuchi, Makoto Nagai, Yasuhito Terui, Mitsuru Nakamura, and Hisashi Yamada‡

From the Division of Hemopoiesis, Institute of Hematology, and the Department of Hematology, Jichi Medical School, Tochigi 329-04, and the 4Department of Internal Medicine (Aoto) and the Department of Molecular Genetics, Institute of DNA Medicine, Jikei University School of Medicine, Tokyo 105, Japan

(Received for publication, August 17, 1996, and in revised form, January 17, 1997)

E2F is a heterodimeric transcription factor that controls transcription of several growth-regulatory genes including cdc2. To investigate the mechanism of interferon-α (IFN-α)-mediated growth suppression of hematopoietic cells, we examined the effect of IFN-α on the expression and function of E2F in IFN-sensitive Daudi cells. Down-regulation of E2F-1, a subunit of E2F, was observed after 8 h of culture with IFN-α; expression of E2F-4, another subunit of E2F, and DP-1, a heterodimeric partner of E2F, was unaffected. Gel shift assays revealed that the DNA binding activity of free E2F, which is composed of E2F-1 and E2F-4, was inhibited by IFN-α. In contrast, IFN-α did not affect the DNA binding ability of E2F-1 and E2F-4 in a complex with retinoblastoma (RB) susceptibility gene family proteins including pRB, p107, and p130. IFN-α could induce dephosphorylation of pRB, thereby turning active E2F-pRB complexes into transcriptional repressors. Transient chloramphenicol acetyltransferase assays revealed that the activity of the E2F-dependent cdc2 promoter was suppressed by IFN-α. These results suggest that the antiproliferative action of IFN-α is mediated through the modulation of E2F activity in two different ways: down-regulation of transcriptionally active free E2F and conversion of E2F-pRB complexes into transcriptional repressors.

Interferons (IFNs) are a family of biological response modifiers with a broad spectrum of action on cellular proliferation as well as immunoregulation (1, 2). It is well known that IFNs can effectively inhibit the growth of some types of hematopoietic cells, and now they have been used as a therapeutic reagent for a variety of hematological malignancies including chronic myeloid leukemia, hairy cell leukemia, and low grade B-cell lymphomas (3). Although IFNs have an established status as a first-line drug for chronic myeloid leukemia and hairy cell leukemia, some problems remain unresolved, including drug resistance and numerous adverse effects. Insights into the molecular mechanisms of IFN action are helpful in resolving these problems. However, the mechanisms regulating the antiproliferative effect of IFNs have not been elucidated fully despite extensive investigations.

Recent investigations revealed that IFNs can induce activation of a group of IFN-stimulated gene products including a double-stranded RNA-activable protein kinase (4), the IFN regulatory factors IRF-1 and IRF-2 (5), gene 200 cluster proteins (6), and RNase L (7). Direct and indirect evidence indicates that several of these proteins may have tumor-suppressive activities. Among the growth-regulatory genes and gene products, IFNs are known to induce down-regulation of c-myc expression (8), dephosphorylation of the retinoblastoma (RB) susceptibility gene product (9), and inhibition of both the expression and activity of cyclin-dependent kinases (10–12). Although the antiproliferative effect of IFN is thought to be mediated through these events, further investigation should be required to elucidate fully the direct mechanisms of its action.

For example, it is difficult to determine whether these events are the cause of the growth arrest or simply a consequence of IFN-induced failure of cell cycle progression. Furthermore, through the study of transcriptional activation in response to IFNs, the direct involvement of Jak-Stat (Janus kinases–signal transducers and activators of transcription) pathways in signal transduction of IFNs has recently been proved (13). The role of the Jak-Stat system in IFN-mediated growth suppression of hematopoietic cells is not yet clarified.

E2F is a heterodimeric transcription factor that was originally identified as an element needed for the E1A-dependent activation of a specific adenoviral E2 promoter (14). E2F is composed of each member of the E2F and DP families. Currently, five distinct E2F family members (E2F-1 to E2F-5) and three DP proteins (DP-1 to DP-3) are known (15). E2F-1 is the most characterized component of E2F and binds to DNA as a heterodimer with DP-1. Although DP-1 itself has little or no affinity for DNA, the association of E2F-1 and DP-1 leads to enhanced DNA binding and is required for E2F site-dependent transcriptional activation (16). E2F binding sites were detected in the promoter regions of many growth-responsive or growth-promoting genes such as c-myc, c-myb, cdc2, and genes for dihydrofolate reductase, thymidine kinase, DNA polymerase-α, and cyclin A (17). The role of E2F in the transcriptional regulation of the c-myc, dihydrofolate reductase, and cdc2 genes has been confirmed (18–20). Moreover, E2F is now considered to be a relevant target of RB family proteins including p107, p130, and RB protein (pRB) itself in their activity as growth suppressors (21). These observations strongly suggest that E2F can be a target of the action of growth-inhibitory factors such as IFNs and transforming growth factor-β.
In this study, with this background in mind, investigations were carried out to clarify the involvement of IFN-α in IFN-induced growth suppression of hematopoietic cells. We have also studied the mechanisms of the inhibition of E2F activity by IFN-α with a special reference to its interaction with RB protein.

MATERIALS AND METHODS

Reagents—Highly purified natural IFN-α derived from Sendai virus-infected Nalawala cells (22) was provided by Sumitomo Pharmaceutical Co. Ltd. (Osaka, Japan). 20 IFN-α components were included in this preparation with specific activities of 1.3 × 10^9–2.8 × 10^10 IU/mg of protein (22).

Cells and Culture—Burkitt lymphoma cell line Daudi was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Cellular DNA and RNA synthesis was monitored by pulse labeling the cells for a final 1 h of the culture with 5 μCi/ml [3H]thymidine and [3H]uridine (Amersham Corp.), respectively. Cell cycle distribution was determined by analyzing the samples of 10^6 cells stained with propidium iodide in a flow cytometry with FACSscan/CellFIT system (Becton-Dickinson, San Jose, CA).

DNA Clones—The following cDNA clones were used in this study: a 1.4-kb EcoRI-BamHI fragment of human E2F-1 cDNA (provided by Drs. William G. Kaelin, Jr., James A. DeCaprio, and David M. Livingston, Dana-Farber Cancer Institute, Boston, MA (23); a 0.9-kb KpnI-PvuII fragment of human cdc2 cDNA (provided by Dr. Paul Nurse, Oxford University, Oxford, U. K.); a 1.8-kb ClaI-EcoRI fragment of c-myc exon 3 cDNA (Oncor Inc., Gaithersburg, MD); and a 2.2-kb EcoRI-BamHI fragment of p33 cDNA (provided by Japanese Cancer Research Resources Bank). A 1.3-kb full-length fragment of human E2F-4 cDNA were generated by reverse transcription-polymerase chain reaction based on the published sequences, respectively (24, 25).

Northern Blotting—Total cellular RNA was isolated by cesium chloride centrifugation using CS120FX ultracetrifuge and SI90AT5 fixed-angle rotor (Hitachi Koki, Co. Ltd.). 10-μg samples were electrophoresed in a 1% agarose gel containing 6% formaldehyde, 20 μg MOPS, 5 mM sodium acetate, and 1 mM EDTA, and blotted onto Hybond N+ synthetic nylon membranes (Amersham Corp.). The membranes were hybridized with each cDNA probe, which was labeled with [32P]dCTP (DuPont-NEN) with the oligonucleotide random priming method.

Western Blotting—Cells were washed with ice-cold TBS buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl), and lysed in EBC buffer (30 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 100 mM sodium fluoride, 200 mM sodium orthovanadate) containing protease inhibitors. An equal amount (150 μg) of the samples was separated on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose filters (Bio-Rad). After blocking in TBS buffer with 4% bovine serum albumin (BSA), the membranes were incubated for 16 h with 1 μg/ml anti-IRB monoclonal antibody PMG3-245 (Pharmingen, San Diego). The membranes were developed as described previously (26). For detection of E2F-1, the membranes were incubated for 16 h with 1 μg/ml of anti-E2F-1-specific monoclonal antibodies KH95 (Santa Cruz Biotechnology, Santa Cruz, CA) and SQ41 (Upstate Biotechnology, Lake Placid, NY). Immune complexes were recovered on protein A-Sepharose beads and washed three times in IP-DOC buffer. The associated proteins were released by the addition of 16 μl of 0.8% deoxycholate and then neutralized with 4 μl of 1% Nonidet P-40 (28). The supernatants (4 μl/assay) were used for E2F gel shift assays as described above.

Transfection and Chloramphenicol Acetytransferase (CAT) Assay—Plasmids were introduced into Daudi cells by electroporation as described previously (29). Briefly, cells (2 × 10^6 cells/transfection) were resuspended in 500 μl of RPMI 1640 medium containing 20% fetal calf serum with 40 μg of linearized plasmid DNA. Electroporation was delivered at 250 V, 960 microfarads by a Gene Pulser (Bio-Rad). The cells were plated on ice for 15 min, resuspended at 5 × 10^5 cells/ml in RPMI 1640 containing 10% fetal calf serum, and split equally into two flasks. IFN-α was added into one of them at the final concentration of 100 IU/ml. Cell extracts were prepared after 24 h of the culture, and the protein concentration was determined by the Bradford method (30). CAT activity was assayed with 50 μg of each sample. CAT assays were carried out according to the standard procedure, and the activity was measured quantitatively by a liquid scintillation counting (30). The 5′-untranslated region of the cdc2 promoter up to nucleotide –383 relative to the transcription start site was linked to the CAT gene in pCAT-basic vector (Promega, Madison, WI) and was used as a reporter plasmid (30). pCAT-control vector (Promega), which contains SV40 promoter and enhancer sequences, was transfected simultaneously and used as a positive control. As a negative control, pCAT-basic vector containing the cdc2 promoter (up to –383) with the mutated E2F binding site was used (30). All plasmids were purified by cesium chloride gradient ultracentrifugation, linearized by appropriate restriction enzymes, and purified again by ethanol precipitation before transfection. Each result was adjusted according to the value obtained with the transfection of pCAT-control vector into corresponding cells.

RESULTS

Effect of IFN-α on Cell Cycle Distribution and DNA Synthesis of Daudi Cells—Among hematopoietic cells, Daudi is the most sensitive to IFN and easily arrests in G0/G1 phase of the cell cycle in response to a relatively low amount of IFN-α (31). We first examined the effect of IFN-α on cell cycle distribution and DNA synthesis of Daudi cells. The cells were seeded at an initial concentration of 5 × 10^5 cells/ml and grown in the absence or presence of 100 IU/ml IFN-α. The change of cell cycle distribution was monitored by serial analysis of the DNA histogram. The proportion of S phase was approximately 45% in untreated Daudi cells (Fig. 1A, T-0). When they were cultured with IFN-α, the S phase fraction was decreased gradually and reached to 21% after 24 h of culture (Fig. 1A, T-24). Conversely, approximately 60% of the cells found to be arrested at S/G1 phase after 24 h. Cellular DNA synthesis, as determined by [3H]thymidine uptake, correlated well with the portion of the cells in S phase, i.e. the S phase was depressed to about 40% of the untreated control at 24 h (Fig. 1B). RNA synthesis was also suppressed by IFN-α in a similar manner (Fig. 1C). Using this culture system, we investigated the effect of IFN-α on E2F.

Effect of IFN-α on mRNA Expression of Major Subunits of DNA Polymerase α
E2F Transcription Factor, E2F-1, E2F-4, and DP-1—Total cellular RNA was isolated from Daudi cells at various time points after IFN-α treatment, and the expression of E2F-1, E2F-4, and DP-1 mRNA was examined by Northern blot hybridization. In agreement with our previous observation (30), both E2F-1 and DP-1 mRNA transcripts were detected readily in untreated Daudi cells, reflecting the proliferative state of the cells. As shown in Fig. 2A, the amount of E2F-1 mRNA decreased significantly after 8 h of culture with IFN-α. It is of note that [3H]thymidine incorporation, a sensitive marker of cell proliferation, was not suppressed at this time point (Fig. 2B). This clearly indicates that down-regulation of E2F-1 mRNA is not a simple consequence of the growth arrest and may have a causative role in IFN-induced growth arrest. In striking contrast, the E2F-4 mRNA level was unchanged during this culture period. DP-1 mRNA expression was also unaffected by the IFN treatment, although a minor decrease was observed after 8 h (Fig. 2A).

Effect of IFN-α on De Novo Synthesis of E2F-1 Protein—Next, we examined the effect of IFN-α on de novo synthesis of the E2F-1 gene product by metabolic labeling and subsequent immunoprecipitation. IFN-treated Daudi cells were pulse labeled for 3 h with [35S]methionine, and whole cell lysates were subjected to immunoprecipitation with anti-E2F-1-specific monoclonal antibodies to monitor the amount of a newly synthesized E2F-1 protein. As a control, the samples were subsequently immunoprecipitated with anti-β-actin antibody. In accordance with the marked reduction of the amount of mRNA transcript, a newly synthesized E2F-1 protein was barely detected after 8 h of the treatment with IFN-α, whereas the amount of β-actin was unaffected (Fig. 3). A minor increase in E2F-1 protein was observed in untreated cells after 8 h of culture. This may be the result of a small increase in the amount of E2F-1 mRNA (see Fig. 2A) during spontaneous cell growth (see Fig. 1B).

Effect of IFN-α on DNA Binding Activity of E2F—To investigate whether this reduction in the amount of E2F-1 protein affects the transcriptional activity of E2F, we first examined the DNA binding ability of E2F in IFN-treated Daudi cells. Nuclear extracts were prepared at various time points after IFN treatment and tested for E2F activity by gel retardation assays using an E2F consensus oligonucleotide from the adenovirus E2 promoter as a probe (32). As shown in Fig. 4A, when the nuclear extract from untreated Daudi cells was used, multiple bands with retarded mobilities were resolved on a 4% native polyacrylamide gel. By oligonucleotide competition assays, seven distinct bands (designated as A to G) were found to be specific E2F complexes. These bands were considered to represent the baseline E2F activity of proliferating Daudi cells. Two other fast migrating bands were considered to be nonspecific, since they were not eliminated by the addition of 100 molar excess of unlabeled E2F oligonucleotide (indicated by stars in Fig. 4). Then we performed antibody perturbation experiments to identify the nature of each complex. Specific antibodies against E2F-1, E2F-4, pRB, p107, and p130 were included in the reaction mixture, and the E2F gel shift assay was carried out as above (Fig. 4B). The results were quantitated by densitometric analysis and are summarized in Table I. Given that any specific antibody against RB family proteins (pRB, p107, and p130) did not affect the intensity of the bands E, F, and G, these complexes represent so-called free E2F. Furthermore, anti-E2F-4- but not anti-E2F-1-specific antibody could eliminate band E completely, indicating that this band is composed mainly of E2F-4 (Fig. 4B). On the other hand, two faster migrating free E2F bands (F and G) were not supershifted by anti-E2F-4 antibody, but the signal intensities were slightly but significantly decreased in the presence of anti-E2F-1 antibody (Fig. 4B and Table I). This suggests that complexes F and G contain E2F-1 and other components of the E2F family such as E2F-2 and E2F-3. Four other bands (A to D) were found to be complexes of E2F and RB family proteins. Anti-pRB antibody effectively supershifted both bands C and D, and the former was eliminated by anti-E2F-4. These results therefore indicate that band C represents a complex containing E2F-4 and pRB. In contrast, band D was not abolished by either anti-E2F-1 or anti-E2F-4, suggesting that other E2F family members such as E2F-2 and E2F-3 form a complex with
Ed as X and Y were observed, and both of them were eliminated completely by the cold competitor (lanes 2 and 6). Anti-E2F-4 antibody could reduce the intensity of band X, indicating that E2F-4 really is present in E2F-pRB complexes (lane 5). Similarly, E2F-1 was shown to be present in band Y (lane 4). There was no difference in the amounts of pRB-bound E2F between pre- and post-treatment samples (compare lanes 1 and 3) despite the decrease in the intensities of E2F-pRB complexes (C and D in Fig. 5A) in gel retardation assays. This suggests that E2F molecules associated with a single pRB molecule are not decreased even after the IFN treatment. This is fully consistent with the previous report by Melamed et al. (36), wherein inhibition of E2F activity started after 8 h of treatment with interleukin-6 or IFN, but it was not eliminated completely even after 24 h. In keeping with the previous assumption that E2F binds preferentially to unphosphorylated pRB (37, 38), this may be explained by the change in phosphorylation status of pRB. Therefore, we then examined the effect of IFN-α on the amount and phosphorylation status of RB protein. This may be also helpful in characterizing further the function of E2F-pRB complexes, since the activity of E2F was regulated mainly by its association with pRB.

Effect of IFN-α on Phosphorylation Status of pRB—Whole cell lysates were prepared from Daudi cells at various time points after the IFN treatment and were subjected to immunoblot analysis for pRB expression. Previous reports have documented that RB protein is unphosphorylated in G1/G0 phase of the cell cycle and is specifically phosphorylated at the G2/M boundary; phosphorylated pRB is dominant throughout the S and G2/M phases (39, 40). Only underphosphorylated RB protein can function as a suppressor of cell growth. These two functionally distinct forms of pRB are clearly distinguishable on a 7.5% polyacrylamide gel.

As shown in Fig. 6A, RB protein was present almost exclusively in heavily phosphorylated forms in untreated Daudi cells, reflecting the active proliferative status of the cells. IFN-α induced the accumulation of an underphosphorylated form of pRB, indicating that pRB is dephosphorylated in response to IFN-α. The complexity of pRB regulation by IFN-α is further demonstrated by the fact that RB protein was also present in a form that is phosphorylated to a lesser extent. This suggests that IFN-α may have a dual effect on pRB, leading to both dephosphorylation and phosphorylation of this protein. Overall, these results indicate that IFN-α modulates the phosphorylation status of pRB, which may contribute to the inhibition of cell proliferation induced by IFN-α.

Effects of IFN-α on E2F Activity—To further investigate the role of IFN-α in the regulation of E2F activity, we performed gel retardation assays using whole cell lysates from Daudi cells after 0, 8, and 24 h of IFN-α treatment. As shown in Fig. 5B, the complex A was found to contain E2F-4, p107, and p130. Complex B disappeared with the addition of anti-p130, but it was not affected by either anti-E2F-1 or anti-E2F-4. Thus, complex B may be composed of p130 and a member of the E2F family other than E2F-1 and E2F-4. E2F-5 is a strong candidate as the E2F molecule present in this complex, as suggested by recent investigations (33, 34).

The effect of IFN-α on E2F activity was analyzed next by comparing the intensities of these bands before and after treatment. Fig. 5A displays the representative result of gel retardation assays with nuclear extracts from Daudi cells isolated after 0, 8, and 24 h of culture with 100 IU/ml IFN-α. The intensity of bands F and G (corresponding to free E2F-1) was decreased significantly by IFN-α. The intensity of bands F and G (corresponding to free E2F-1) was decreased to approximately 25% of the untreated control by densitometric comparison. The abundance of the band E, which corresponds to free E2F-4, was unaltered at 8 h but was reduced markedly after 24 h of treatment (Fig. 5A). IFN-α also reduced the amounts of E2F-pRB complexes (C and D) and the E2F-4-p107 complex (A). The decrease in the E2F-p107 complex may correspond to the reduction of S phase cells by IFN, since this complex was reported to be preferentially formed in S phase (35).

Next, we performed an immunoprecipitation-deoxycholate release assay to confirm that E2F-4 really made a complex with pRB and also to see more precisely the effect of IFN on the E2F-4-pRB complex. pRB-associated proteins were immunoprecipitated from whole cell lysates of Daudi cells before and after the IFN treatment, and E2F gel shift assays with antibody perturbation were carried out after deoxycholate release of the supernatants. As shown in Fig. 5B, two bands (designated as X and Y) were observed, and both of them were eliminated completely by the cold competitor (lanes 2 and 6). Anti-E2F-4 antibody could reduce the intensity of band X, indicating that E2F-4 really is present in E2F-pRB complexes (lane 5). Similarly, E2F-1 was shown to be present in band Y (lane 4). There was no difference in the amounts of pRB-bound E2F between pre- and post-treatment samples (compare lanes 1 and 3) despite the decrease in the intensities of E2F-pRB complexes (C and D in Fig. 5A) in gel retardation assays. This suggests that E2F molecules associated with a single pRB molecule are not decreased even after the IFN treatment. This is fully consistent with the previous report by Melamed et al. (36), wherein inhibition of E2F activity started after 8 h of treatment with interleukin-6 or IFN, but it was not eliminated completely even after 24 h. In keeping with the previous assumption that E2F binds preferentially to unphosphorylated pRB (37, 38), this may be explained by the change in phosphorylation status of pRB. Therefore, we then examined the effect of IFN-α on the amount and phosphorylation status of RB protein. This may be also helpful in characterizing further the function of E2F-pRB complexes, since the activity of E2F was regulated mainly by its association with pRB.

Effect of IFN-α on Phosphorylation Status of pRB—Whole cell lysates were prepared from Daudi cells at various time points after the IFN treatment and were subjected to immunoblot analysis for pRB expression. Previous reports have documented that RB protein is unphosphorylated in G1/G0 phase of the cell cycle and is specifically phosphorylated at the G2/M boundary; phosphorylated pRB is dominant throughout the S and G2/M phases (39, 40). Only underphosphorylated RB protein can function as a suppressor of cell growth. These two functionally distinct forms of pRB are clearly distinguishable on a 7.5% polyacrylamide gel.

As shown in Fig. 6A, RB protein was present almost exclusively in heavily phosphorylated forms in untreated Daudi cells, reflecting the active proliferative status of the cells. IFN-α induced the accumulation of an underphosphorylated
form of pRB after 24 h of treatment (Fig. 6A). Recently, Weintraub et al. (41) reported that unphosphorylated RB protein, upon binding to E2F, switches E2F from a transcriptional activator to a repressor. Thus, E2F-pRB complexes present after the IFN treatment may function as a suppressor of cell growth. Taken together, these results indicate that IFN-α suppresses transcriptional activity of E2F in two ways: through reduction of transcriptionally active free E2Fs (E2F-1 and E2F-4) and through induction of unphosphorylated pRB to change E2F-pRB complexes to transcriptional repressors.

**Effect of IFN-α on E2F-dependent transcription of the cdc2 Gene**—We next investigated the effect of IFN-α on the trans-activating ability of E2F in vivo. For this purpose, we chose to examine whether IFN-α could suppress the activity of the cdc2 promoter by transient CAT assay. A 467-base pair fragment of the 5′-flanking region of the cdc2 gene (–383 to +87) was linked to the CAT gene in pCAT-basic vector and used as a reporter plasmid (hereinafter designated as pCAT-5′-cdc2). In our previous study, it was shown that this segment had a strong promoter activity that was dependent on the binding of E2F at nucleotide positions −212 to −117 (30). A nonbinding mutation was introduced into the E2F binding site of pCAT-5′-cdc2 (5′-TTTCGCGC-3′ to 5′-TTTCGATC-3′) and used as a negative control. The plasmid containing SV40 promoter-driven CAT (pCAT-control) was transfected simultaneously into corresponding cells and used as a positive control. These
plasmids were transiently transfected into Daudi cells by electroporation, and the cells were split equally into two flasks. IFN-α was added into one of them, and the cells were cultured for 24 h and harvested for measurement of intracellular CAT activity. The promoter activity of pCAT-control was independent of E2F and was shown to be unaffected by IFN-α (data not shown). In contrast, IFN-α could suppress the CAT activity of pCAT-5'-cd2 to 74 ± 4% of the untreated control in four independent experiments (p < 0.01 by Student’s t test, Fig. 7, left panel). As anticipated, IFN-α could not inhibit the promoter activity of the mutant plasmid lacking a functional E2F binding site (Fig. 7, right panel). This indicates that E2F-dependent promoter activity was regulated negatively by IFN-α in vivo. This result facilitated further examination of the expression of the genes whose promoter contains functional E2F binding sites in IFN-treated Daudi cells.

IFN-α Could Suppress the Expression of c-myc and cdc2 mRNA—Both c-myc and cdc2 had functional E2F binding sites in their promoter regions, and their transcription was known to be regulated by E2F (18, 20, 30). The effect of IFN-α on expression of c-myc and cdc2 mRNAs was investigated in IFN-treated Daudi cells. Expression of p53 mRNA, whose transcription is independent of E2F, was examined simultaneously as a negative control. As shown in Fig. 8A, IFN-α could effectively inhibit the expression of c-myc and cdc2, whereas p53 mRNA expression was unaffected. IFN-induced suppression of cdc2 mRNA occurred after 12 h of treatment, whereas down-regulation of c-myc mRNA became evident after 72 h. The inhibition of cdc2 and c-myc mRNA was apparently preceded by the suppression of E2F activity. This is entirely compatible with the notion that IFN-induced suppression of these growth-related genes is mediated at least in part through the inhibition of E2F activity. Down-regulation of these genes may contribute somewhat to the growth arrest of Daudi cells, although many other factors should be involved in this process (Fig. 8B).

DISCUSSION

Two decades ago the antiproliferative activity of IFNs was first identified (42). However, the mechanism of IFN-mediated growth suppression is not fully understood. Previous investigations have shown that IFN causes G0/G1 arrest through the reduction of c-myc proto-oncogene (8) and dephosphorylation of pRB (9). Regarding the cell cycle-regulatory elements, down-regulation of cyclin A and cyclin E- and cyclin D1-dependent cdk2 kinase activity was also reported (12). Although the action of IFN is thought to be mediated through these events, it is not clear whether or not this is a direct action. It is possible that some of these events were the result of IFN-induced growth arrest. Given the fact that IFN is now used widely as a therapeutic reagent, further investigation should be required to elucidate fully the direct mechanism of its action for better clinical application.

In this work, we investigated the effect of IFN-α on tran-
Modulation of E2F Activity by Interferon

Fig. 7. Inhibition of the E2F-dependent cdc2 promoter activity by IFN-α.

A 467-base pair fragment of the 5′-flanking region of the cdc2 gene (−383 to +87) was linked to the CAT gene in pCAT-basic vector (Wild Type) and transfected into Daudi cells by electroporation. A nonbinding mutation was introduced into the E2F site of the same plasmid and used as a negative control (Mutant). The cells were split equally into two fractions, and IFN-α (100 IU/ml) was added into one of them (+). Cell extracts were prepared after 24 h, and 50 μg of each sample was used for CAT assay. Relative CAT activity was calculated as the percentage of the value obtained in the absence of IFN (−). The mean ± S.D. (bar) of four independent experiments is shown. Statistical analysis was performed with Student’s t test.

Fig. 8. Effect of IFN-α on expression of c-myc and cdc2 mRNA transcripts in Daudi cells. Panel A, Daudi cells were cultured in the absence (−) or presence (+) of 100 IU/ml IFN-α, and total RNA was isolated after 0, 24, 48, and 72 h. The expression of c-myc, cdc2, and p53 mRNA transcripts was analyzed by Northern blotting. Panel B, the growth-inhibitory effect of IFN-α was monitored simultaneously by [3H]thymidine uptake as described in the legend of Fig. 1. Data shown as representative of three independent experiments.
E2F inhibits E2F-dependent transcription (37, 38). As cells pass the G1/S boundary by growth stimulation, pRB becomes phosphorylated, and E2F is released. The unbound free E2F (mainly E2F-1 and E2F-4) is presumed to be transcriptionally active. Conversely, some negative growth factors such as transforming growth factor-β can induce dephosphorylation of pRB, thereby inhibiting E2F activity and leading cells to G1 arrest (26, 51). The accumulation of the phosphorylated, growth-suppressive form of RB protein and its negative effect on E2F activity might be important for IFN-mediated growth suppression. In this study, we demonstrated that E2F-4, in addition to E2F-1, formed a specific complex with pRB in Daudi cells. E2F-4 is a recently identified member of the E2F family which makes a complex with RB family proteins (25). E2F-4-p107 and E2F-4-p130 complexes are present in early to mid G1 phase of the cell cycle and are shown to act as repressors of E2F site-directed transcription of B-myb (52) and cdc2 (53). In previous reports, E2F-4 was shown to bind preferentially p107 and p130 and was believed not to bind pRB (43, 44). This is somewhat contradictory to our present observation that E2F-4 is present in a complex with pRB. However, Ikedo et al. (54) recently described that E2F-4 was the major species of E2F in quiescent fibroblasts and in differentiated HL-60 cells, where E2F-4 made complexes with unphosphorylated pRB and p130 to induce cell cycle arrest. They claimed that it is the ratio of free E2F to pRB-bound E2F which is critical in the decision to pass through the G1/S transition. Our finding is fully consistent with their assertion and demonstrates that the relative increase in the ratio of pRB-bound E2F to free E2F, mainly because of the decrease in free E2F, is linked to IFN-induced suppression of E2F-mediated transcription and cell cycle arrest.

How does IFN-α repress E2F-1 mRNA expression? Transcriptional activation in response to IFN-α is known to be mediated through the activation of a multiprotein DNA-binding complex described as IFN-stimulated gene factor 3 (ISGF3) (55). The components of ISGF3 were purified and are recognized now as Stat1, Stat2, and p48 (56). Tyrosine phosphorylation of Stat proteins by IFN-α/β receptor-associated Jak kinases is necessary for activation of ISGF3. The phosphorylated Stat proteins move to the nucleus and bind specific DNA elements called ISGF3-stimulated response elements to direct transcription (57). However, there is no binding site for ISGF3 in the promoter region of the E2F-1 gene according to recent reports (58, 59). Therefore, direct involvement of the Jak-Stat signaling pathway in IFN-induced suppression of E2F-1 mRNA transcription is unlikely. Recent investigations suggest that transcription of the E2F-1 gene is under autoregulatory control through the E2F binding sites in its promoter (58, 59). Accord-ingly, transcription of E2F-1 is repressed when E2F binds to the promoter region of the E2F-1 gene in a complex with underphosphorylated pRB, since this complex acts as a transcriptional repressor. Although this is a plausible explanation, down-regulation of E2F-1 mRNA started apparently prior to dephosphorylation of pRB in our experiments. It is possible that other molecules in the E2F family play a role in IFN-mediated inhibition of E2F-1 mRNA. E2F-5 may be a good candidate because it binds to the RB family protein p130 and is the predominant E2F complex in the early phases of the cell cycle (G1 to mid G1) (34, 35). To understand the precise mechanism of the repression of E2F-1 expression requires a study of the effect of IFN-α on other members of the E2F family. Detailed analysis of these subjects is currently under way in our laboratory.

Finally, we have to mention the discrepancy between our study and the previous one by Melamed et al. (36) regarding the expression patterns of c-Myc after IFN treatment. As shown in Fig. 8, down-regulation of c-myc mRNA was observed after 72 h of IFN treatment in our study. However, they described that c-Myc protein levels were inhibited at 8 h in Daudi cells (36). Thus, we also performed Western blot analysis of c-Myc expression in IFN-treated Daudi cells. Consistent with their result, the c-Myc protein was down-regulated after 24 h of culture with IFN in our system (data not shown), whereas c-myc mRNA expression was unaffected at this time point. This suggests that there might be multiple distinct mechanisms for IFN-induced suppression of c-Myc including E2F-dependent inhibition of mRNA transcription and post-translational modification of the protein levels. The precise mechanism of the latter is at present unknown.
Modulation of E2F Activity by Interferon

42. Knight, E. (1976) Nature 263, 302–304
43. Beijerbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlée, L., Voorhoeve, P. M., and Bernards, R. (1992) Genes Dev. 6, 2680–2690
44. Vairo, G., Livingston, D. M., and Ginsberg, D. (1995) Genes Dev. 9, 869–881
45. Johnson, D. G., Schwarz, J. K., Cress, W. D., and Nevins, J. R. (1993) Nature 365, 349–352
46. DeGregori, J., Kowalik, T., and Nevins, J. R. (1995) Mol. Cell. Biol. 15, 4215–4224
47. Schwarz, J. K., Bassing, C. H., Kovesdi, I., Datto, M. B., Blazing, M., George, S., Wang, X., and Nevins, J. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 483–487
48. DeGregori, J., Leone, G., Ohtani, K., Miron, A., and Nevins, J. R. (1995) Genes Dev. 9, 2873–2887
49. Guy, C. T., Zhou, W., Kaufman, S., and Robinson, M. O. (1996) Mol. Cell. Biol. 16, 685–693
50. Qin, X.-Q., Chittenden, T., Livingston, D. M., and Kaelin, W. G. (1992) Genes Dev. 6, 953–964
51. Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M., and Massagué, J. (1990) Cell 62, 175–185
52. Zwickert, J., Liu, N., England, K., Lucibello, F. C., and Müller, R. (1996) Science 271, 1595–1597
53. Temmassi, S., and Pfeifer, G. P. (1995) Mol. Cell. Biol. 15, 6901–6913
54. Ikeba, M., Jakoi, L., and Nevins, J. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3218–3220
55. Levy, D. E., Kessler, D. S., Pine, R., and Darnell, J. E. (1989) Genes Dev. 3, 1362–1371
56. Schindler, C., Fu, X.-Y., Improti, T., Aebersold, R., and Darnell, J. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7836–7840
57. Schindler, C., Shi, K., Prezioso, V. R., and Darnell, J. E. (1992) Science 257, 809–813
58. Johnson, D. G., Ohtani, K., and Nevins, J. R. (1994) Genes Dev. 8, 1514–1525
59. Neuman, E., Flemington, E. K., Sellers, W. R., and Kaelin, W. G. (1994) Mol. Cell. Biol. 14, 6607–6615
60. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
Modulation of E2F Activity Is Linked to Interferon-induced Growth Suppression of Hematopoietic Cells
Satsuki Iwase, Yusuke Furukawa, Jiro Kikuchi, Makoto Nagai, Yasuhito Terui, Mitsuru Nakamura and Hisashi Yamada

J. Biol. Chem. 1997, 272:12406-12414.
doi: 10.1074/jbc.272.19.12406

Access the most updated version of this article at http://www.jbc.org/content/272/19/12406

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 37 of which can be accessed free at http://www.jbc.org/content/272/19/12406.full.html#ref-list-1