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IFNα induces the expression of the cyclin-dependent kinase inhibitor p21 in human prostate cancer cells

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Prostate cancer, like other types of cancer, is associated with the loss of cell cycle control, resulting in unregulated growth of cells. We report here on the inhibitory effects of interferon α (IFNα) on the cell cycle of prostate cancer cells, using the human prostate carcinoma cell line DU145 that has mutations in the tumor suppressor genes pRB, p53 and KAI1. IFNα inhibited growth and colony formation of DU145 cells and analysis by flow cytometry suggests that IFNα inhibited the progression of these cancer cells from the G_1 through S phase of the cell cycle. IFNα treatment of DU145 cells reduced cyclin dependent kinase 2 (cdk2) activity. In particular, cyclin E dependent cdk2 activity was inhibited by IFNα treatment. IFNα treatment, however, did not affect the amount of cdk2 bound to cyclin E. Consistent with this data, IFNα was able to induce expression of the kinase inhibitor p21 in DU145 cells. Furthermore, IFN treatment increased the amounts of p21 complexed with cdk2 in these cells. These data support a role for p21 in mediating the antiproliferative action of IFNα. The induction of p21 and its growth inhibitory effects in DU145 cells appears independent of p53, pRB and KAI1 status.

Keywords: Interferon α; prostate cancer; cyclin dependent kinase 2; cyclin E; p21

Introduction

Prostate cancer is the most commonly diagnosed malignancy in men (Parker *et al*., 1996). Like other cancers, prostate cancer results from a loss or mutation of regulatory factors of the cell cycle such as oncogenes and tumor suppressor genes (Garnick, 1994; Cavenee and White, 1995). The type I IFNs such as IFNα have been shown to possess antiproliferative effects on a variety of normal and tumor cell lines in culture and *in vivo* (Creasey *et al*., 1980; Pontzer *et al*., 1991; Gutterman, 1994). Previous studies have shown that human prostate cancer cell lines are also sensitive to the antiproliferative properties of type I IFNs (Sica *et al*., 1989; Nakajima *et al*., 1994). However, although the anticellular effects of IFNs on prostate cancer cells have been recognized, the mechanism behind this cellular inhibition remains unclear.

The type I IFNs appear to exert their anticellular effects on many cell types by blocking progression of cells through the G_1 phase of the cell cycle and/or from the G_1 into the S phase of the cell cycle (Creasey *et al*., 1980; Roos *et al*., 1984; Tamm *et al*., 1987; Pontzer *et al*., 1991). In this report, we examine the effect of IFNα on cell growth of a human prostate cancer cell line, DU145. The DU145 cell line has multiple defects in genes encoding for the tumor suppressors pRB, p53 and KAI1 (Bookstein *et al*., 1990; Isaacs *et al*., 1991; Dong *et al*., 1995). In this background, IFNα still inhibited the progression of these cells from the G_1 into the S phase. Consistent with this, IFNα inhibited the activity of a cyclin dependent kinase (cdk), cdk2, that is associated with this stage of the cell cycle (Tsai *et al*., 1993; Pines and Hunter, 1995). In particular, cyclin E-associated cdk2 activity that is required for DNA synthesis and for progression into the S phase of the cell cycle (Ohtsubo *et al*., 1995), was found to be reduced. Furthermore, the kinase inhibitor p21, which is known to bind the cyclin E-cdk2 complex and block its kinase activity (Pines and Hunter, 1995), was found to be induced by IFNα treatment. These findings describe a mechanism by which IFNs regulate tumor cell growth in a cell line that possesses multiple tumor suppressor gene mutations.

Results

We began our investigation into the effects of IFNα on prostate cancer cells by determining the antiproliferative properties of IFNα on DU145 cells, a human prostate cancer cell line. IFNα inhibited colony formation of DU145 cells at low cell density in a dose dependent manner as shown in Table 1. We also determined the antiproliferative effects of IFNα by utilizing direct cell counts. DU145 cells were treated with 2500 units/ml of IFNα and the overall reduction in cell number was determined (Figure 1). A reduction in the rate of growth by approximately 50% was observed in IFN treated cultures versus untreated cultures. Thus, IFNα has antiproliferative activity on DU145 cells.

In examining the effects of IFNα on the DU145 cell cycle, we first looked at the incorporation of [^{3}H]thymidine by cultures synchronized, by serum starvation, into G_0/G_1 (Figure 2). The incorporation of thymidine by cells is a measure of chromosomal replication and is therefore an indication of cellular activity in the S phase of the cell cycle (Tamm *et al*., 1987). DU145 cells were incubated with or without 2500 units/ml IFNα for 16, 20 or 24 h and pulse-labeled at each time point with [^{3}H]thymidine. At 16 h the amount of [^{3}H]thymidine incorporated by IFN treated cells was only 29% of the incorporation seen with the untreated cells. At 20 and 24 h IFN treated cells incorporated about 60% of the [^{3}H]thymidine incorporated by untreated cells. Thus, IFN treated cells entered the S phase at least 4 h later than untreated

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Table 1 IFNα inhibition of colony formation of DU145 cells[a]

IFNα (units/ml)	*Colonies/well (means±s.d.)*	*Inhibition (%)*
0	77.7±4.2	–
625	60.3±4.7	22.4
1250	40.3±3.5	48.1
2500	33.7±3.2	56.6
5000	30.6±2.1	60.6

[a]DU145 cells were plated at 600 cells/well with various doses (units/ml) of IFNα for 6 days and subsequently stained with crystal violet. Samples were assessed in triplicate and results are expressed as the mean number of colonies±s.d. Statistical significance was shown by Student's *t*-test between the number of colonies in the presence or absence of 625 U/ml ($P<0.05$), 1250 U/ml ($P<0.02$), 2500 U/ml ($P<0.006$) and 5000 U/ml ($P<0.006$) of IFNα

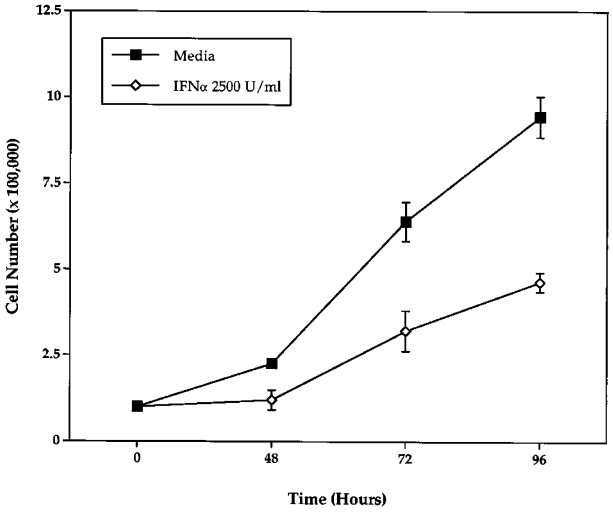

Figure 1 IFNα inhibits cellular proliferation of DU145 cells. Synchronized DU145 cells (1×10^5/well) were incubated with or without 2500 units/ml IFNα for 48, 72, or 96 h. Wells were then harvested and the total number of live cells determined. Data are expressed as total number of cells per sample±s.d. Statistical significance was shown by Student's *t*-test between the number of cells in the presence and absence of 2500 U/ml IFNα for 48 ($P<0.03$), 72 ($P<0.04$) and 96 ($P<0.03$) hours

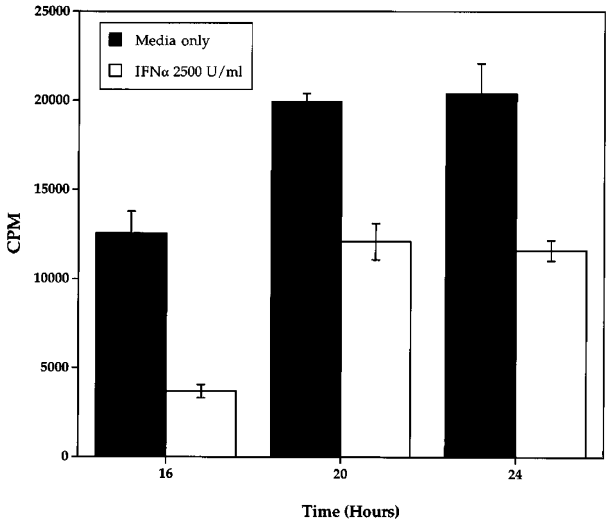

Figure 2 Treatment of DU145 cells with IFNα inhibits [^{3}H]thymidine incorporation. DU145 cells (1×10^5) synchronized in G_0/G_1 were incubated with or without IFNα for 16, 20, or 24 h. Cells were harvested and reseeded into 96 well plates at 2.5×10^4 cells/well, incubated with [^{3}H]thymidine for 2 h and harvested on a cell harvester. Data are expressed as mean c.p.m.±s.d. Statistical significance was shown by Student's *t*-test between [^{3}H]thymidine incorporation by cells in the presence and absence of 2500 U/ml IFNα for 16 ($P<0.0006$), 20 ($P<0.0003$) and 24 ($P<0.0006$) hours

Table 2 Cell cycle analysis of IFNα treated DU145 cells[a]

Time (h)	*IFNα (units/ml)*	*Cell cycle phase (%)* G_0/G_1	*S*	G_2/M
0	0	63.0	18.5	18.5
16	0	54.9	33.6	11.5
	2500	66.9	22.1	11.0
20	0	39.5	46.2	14.2
	2500	57.8	30.9	11.3
24	0	39.2	35.4	25.4
	2500	53.8	33.7	12.5
40	0	62.1	24.9	13.0
	2500	48.7	32.7	18.6

[a]DU145 cells were treated with 0 or 2500 units/ml for 0, 16, 20, 24, or 40 h. Progression through the cell cycle was examined using propidium iodide staining. Data are presented as percentage of cells in each stage of the cell cycle

cells. Consistent with this, at 20 and 24 h, IFN treated cells reached a level of [^{3}H]thymidine incorporation seen with untreated cells at 16 h. These data suggest that IFNα inhibited the progression of DU145 cells from G_1 through S phase.

The inhibitory effects of IFNα on the DU145 cell cycle were further examined using flow cytometry analysis (Table 2, Figure 3). DU145 cells synchronized by serum starvation into G_0/G_1 were stimulated to enter the cell cycle by serum addition in the presence or absence of 2500 units/ml IFNα. As can be seen from Table 2, untreated cells rapidly advanced through the G_0/G_1 and S phases, and by 40 h cells had already completed one full cycle and were again entering the S phase. IFN treated cells, however, progressed more slowly. After 24 h 54% of the IFN treated cells were still in the G_0/G_1 phase, while untreated cells had only 39% of cells in G_0/G_1. The flow cytometry histograms (Figure 3) depict the state of the DU145 cell cycle at 16, 20 and 24 h in the presence and absence of IFN. A similar pattern was seen with IFN treatment at 1000 units/ml (data not shown). Consistent with the [^{3}H]thymidine incorporation by IFNα treated DU145 cells, flow cytometry analysis showed that IFNα inhibited progression of prostate cancer cells through G_1 and early S phase of the cell cycle.

In order to determine the relationship of the phase of the cell cycle specifically inhibited by IFN in the context of cyclin dependent kinase (cdk) activity, we looked at the activity of a cdk, cdk2, that is active during the G_1 and S phases of the cell cycle (Pines and Hunter, 1995). DU145 cells were cultured with or without IFN for 16 or 24 h and subsequently harvested. For each time point, cdk2 was immunoprecipitated and function assessed by histone H1-dependent kinase activity (Table 3). DU145 cells synchronized to G_0/G_1 had low cdk2 activity at 16 h. However, by 24 h these cells increased their cdk2 activity by greater than tenfold, while IFN treated cells showed only a fourfold increase, resulting in a 74% reduction of cdk2 activity over the control. The data show that IFNα is able to reduce the activity of a cdk

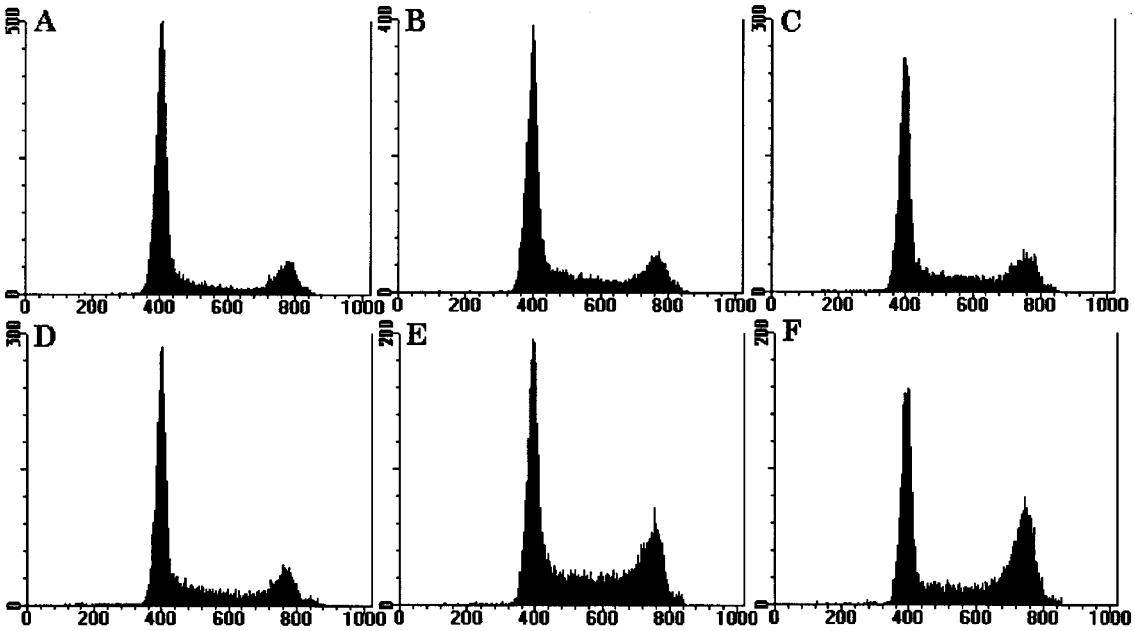

Figure 3 IFNα inhibits the progression of DU145 cells through G_1 and S phase of the cell cycle. DU145 cells were synchronized in G_0/G_1 by growing in media containing 0.5% FBS. Progress through the cell cycle was examined using propidium iodide. Horizontal axis: relative fluorescence intensity: vertical axis: number of cells. (**A**), (**B**) and (**C**) IFNα treated cells (2500 units/ml); (**D**), (**E**) and (**F**) untreated controls, at 16, 20 and 24 h after the initiation of culture, respectively

Table 3 Effects of IFNα treatment on cdk2 activity[a]

Time (h)	*IFNα (units/ml)*	*CPM*	*reduction (%)*
16	0	8042	–
	2500	6138	23.7
24	0	94880	–
	2500	24570	74.1

[a]DU145 cells were treated with 2500 units/ml IFNα for 16 or 24 h. Cyclin dependent kinase 2 activity was assessed by histone H1-dependent kinase activity. Cdk2 activity is represented as c.p.m. with corresponding percent reduction. Similar data with the same patterns were observed in three repeats of this experiment

Table 4 Effect of IFNα treatment on cyclin specific cdk2 activity[a]

Cyclin	*Time (h)*	*IFNα (units/ml)*	*CPM*	*Reduction (%)*
Expt 1				
E	16	0	29507	–
		2500	22861	22.5
	24	0	28607	–
		2500	17742	37.9
D1	16	0	5321	–
		2500	3881	27.1
	24	0	4642	–
		2500	5291	–
Expt 2				
E	16	0	15954	–
		2500	11761	26.3
	24	0	14296	–
		2500	9907	30.1
D1	16	0	4786	–
		2500	4936	–
	24	0	5666	–
		2500	7818	–

[a]DU145 cells were treated with 0 or 2500 units/ml IFNα for 16 or 24 h. The cyclin-cdk2 complex was immunoprecipitated with antibodies specific for either cyclin E or cyclin D1. Cdk2 activity was then assessed by histone H1-dependent kinase activity. Cdk2 activity is represented as c.p.m. with corresponding percent reduction

specific for the G_1 and S phases of the cell cycle in prostate cancer cells and thus inhibit the progression through the cell cycle.

To further examine the stage of the cell cycle that is regulated by IFNα, we looked at the cyclin specificity of the inhibition of cdk2 activity. Cdk2 binds cyclin D1 and cyclin E during the G_1 phase and G_1 to S phase transition, respectively (reviewed in Pines and Hunter, 1995). Cell lysates from DU145 cells treated with IFNα for 16 or 24 h were immunoprecipitated using antibodies specific for cyclin D1 or cyclin E and cdk2 activity was subsequently assessed (Table 4). IFNα treated cells showed up to a 38% reduction of cyclin E-cdk2 activity over the control, but did not show consistent inhibition of cyclin D1-cdk2 activity. Of the 74% IFNα induced reduction in overall cdk2 activity (Table 3), 38% is apparently due to the reduction of cyclin E dependent cdk2 activity (Table 4). The remaining inhibition of cdk2 activity by IFNα is probably due, at least in part, to inhibition of activity in the cyclin A-cdk2 complex (Tiefenbrun *et al*., 1996).

We next immunoprecipitated cyclin E and immunoblotted using a cdk2 antibody in order to determine relative amounts of cdk2 complexed to cyclin E. Figure 4 shows that IFN treatment of cells did not significantly affect the levels of cdk2 complexed with cyclin E. Thus, IFNα inhibition of the activity of the cyclin E-cdk2 complex did not affect the formation of the cyclin E-cdk2 complex in DU145 cells.

These results suggested that a kinase inhibitor may be responsible for IFNα induced decrease in cyclin E-

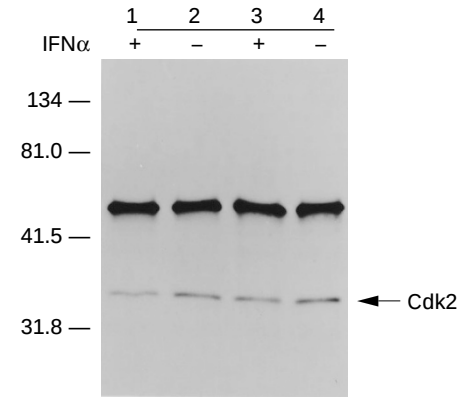

Figure 4 IFNα does not affect cyclin E-cdk2 complex formation in DU145 cells. The amount of cdk2 complexed with cyclin E was determined by immunoprecipitation of DU145 cell lysates with cyclin E antibodies and immunoblotting with antibodies specific for cdk2, as described in Materials and methods. Lanes 1 and 2 represent lysates from cells treated for 16 h with 2500 units/ml IFNα or media alone, respectively. Lanes 3 and 4 are from cells treated for 24 h with or without IFN, respectively

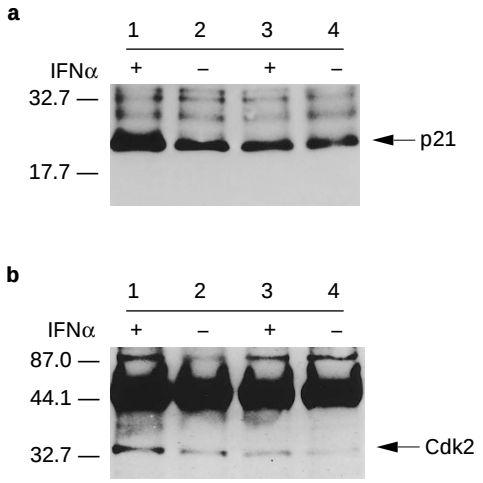

Figure 5 (**a**) IFNα treatment increases and/or maintains p21 levels in synchronized DU145 cells. Cell lysates were immunoprecipitated and immunoblotted using p21 antibodies, as described in Materials and methods. Lanes 1 and 2 represent lysates from synchronized cells treated with 2500 U/ml IFNα or media alone, respectively, for 16 h. Lanes 3 and 4 are from cells treated for 24 h with or without IFN, respectively. The percent decrease in p21 levels for lanes 1, 2, 3 and 4 was 40.1%, 62.7%, 68.2% and 70.0%, respectively, as determined using densitometric scanning of radiographic film. The percent decrease represents the ratios of band intensities from DU145 cell lysates at initiation of cultures (0 h, data not shown) and lanes 1 through 4. (**b**) Cdk2 levels correspond to p21 expression in IFNα treated cells. The immunoblot from (**a**) was reanalysed using antibodies specific for cdk2. The lane assignments are as stated for (**a**). The percent decrease in cdk2 protein levels was 64.3%, 81.5%, 83.2% and 91% for lanes 1, 2, 3 and 4 respectively, as determined by densitometric scanning as described for (**a**)

cdk2 activity. The kinase inhibitor p21 is known to bind cyclin E-cdk2 and block its activity (reviewed in Pines and Hunter, 1995). It is also known that serum starvation of cells increases p21 levels and restimulation by serum causes p21 turnover and reduction in p21 levels (Pines and Hunter, 1995). We therefore used DU145 cells synchronized by serum starvation into G_0/G_1 to determine whether p21 is involved in inhibiting the G_1 to S phase transition in IFN treated cells. Cell lysates from DU145 cells treated with IFNα for 16 and 24 h were immunoprecipitated using p21 antibodies and the presence of both p21 and cdk2 in these samples was subsequently assessed by immunoblotting. As shown in Figure 5a, lysates from cells treated with IFN had greater expression of p21 than untreated cells. In the same immunoprecipitates, at 16 and 24 h, cdk2 protein levels were also higher in IFN treated cells than in untreated cells (Figure 5b), supporting the conclusion that in IFN treated cells increased levels of p21 were associated with cdk2. This suggests that the expression of p21 in IFN treated cells played an important role in inhibiting the cdk2 activity in these cells.

To establish that IFN treatment induces the expression of p21, cell lysates from asynchronous DU145 cells that were treated with IFNα or media alone were assessed for p21 as described above. IFNα treatment progressively induced the expression p21 (Figure 6a) over that seen in untreated cells. Densitometric analysis (Figure 6b) of the p21 bands in Figure 6a showed that IFN treated cells had approximately twice the levels of p21 compared to untreated cells. Thus, IFNα inhibits the G_1 to S phase transition of the cell cycle by inducing p21 expression in prostate cancer cells. In similar experiments to address the role of two other kinase inhibitors, p27 and p16, we did not see induction of p27 or p16 expression by IFN treatment of DU145 cells (data not shown) suggesting a unique role for p21 in these effects. Thus, IFNα inhibits the G_1 to S phase transition of the cell cycle by inducing p21 expression in a prostate cancer cell line.

Discussion

Type I IFNs are potent regulators of cell growth and proliferation for a variety of cell types and have been shown to decrease the rate of progression of cells through the cell cycle. Our examination of the ability of IFNα to inhibit the growth of DU145 cells reveals that IFNα can specifically affect the prostate cancer cell cycle at the G_1 to S phase transition. This inhibition of cell replication is at least partly due to an increase in p21 expression and the subsequent decrease in activity of the cyclin E-cdk2 complex. Although we did not see induction of expression of other kinase inhibitors like p27 or p16 by IFNα, it is possible that IFNα can effect multiple regulators and inhibitors that influence the cell cycle of tumor cells. Since the mechanism behind IFNα regulation of the cell cycle is not well characterized, understanding which cell cycle proteins are turned on or off in prostate cancer cells provides insight into IFN regulation of tumor cells in general.

Many of the proteins involved in controlling the cell cycle have been designated as products of tumor suppressor genes or oncogenes due to the association of mutations of these genes with different types of cancers. Various oncogenes and tumor suppressor genes have been identified in prostatic tumors including mutations in p53 and pRB (Netto and Humphrey, 1994). In this regard, the human DU145 cell line provides an interesting model for studying the

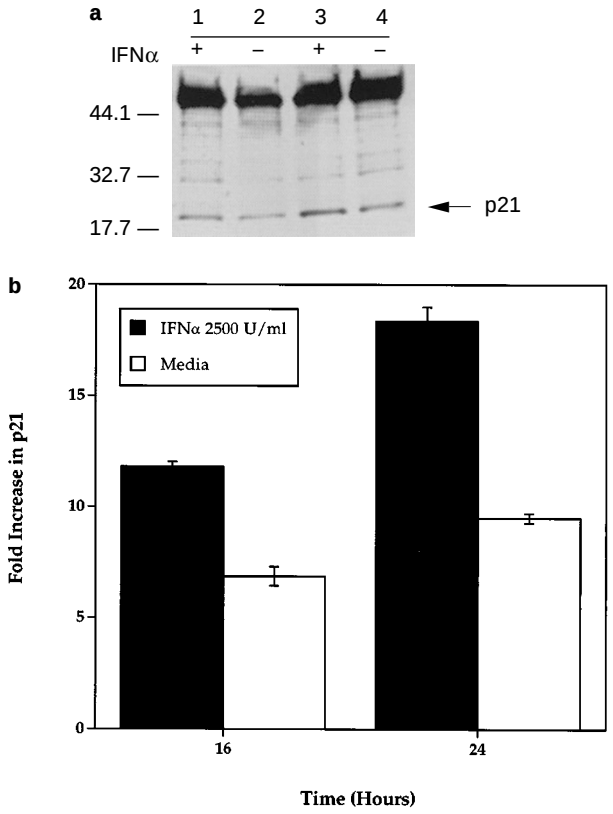

Figure 6 (**a**) IFNα induces p21 expression in DU145 cells. Cell lysates from DU145 cells were immunoprecipitated and immunoblotted with antibodies specific for p21. Lanes 1 and 2 represent lysates from cells treated for 16 h with 2500 U/ml IFNα or media alone, respectively. Lanes 3 and 4 represent lysates from cells treated for 24 h with or without IFN, respectively. (**b**) The fold increase in p21 levels for 16 and 24 h in IFN treated and untreated cells was determined by densitometric scanning of radiographic film. The fold increase represents the ratios of band intensities from DU145 cell lysates at initiation of cultures (0 h, data not shown) and Lanes 1 through 4

effects of type I IFNs on tumor cells because DU145 cells have multiple genetic defects including abnormal p53, retinoblastoma (RB) and KAI1 tumor suppressor proteins (Bookstein *et al*., 1990; Isaacs *et al*., 1991; Dong *et al*., 1995). By using the DU145 cell line, we have shown here that IFNs are potent antiproliferative agents against tumors even with these genetic defects and characteristics. Thus, IFNs can affect tumor cells independent of their RB protein (pRB) or p53 status.

It is, however, likely that IFNs influence multiple pathways that may or may not overlap, since IFNα has previously been shown to suppress the phosphorylation of pRB (Thomas *et al*., 1991; Burke *et al*., 1992; Kumar and Atlas, 1992; Resnitzky *et al*., 1992). Much of the previous work looking at the effects of type I IFNs and their effects on the cell cycle have used the Daudi cell line (Bybee and Thomas, 1992; Resnitsky *et al*., 1992; Kumar and Atlas, 1992). Daudi cells are commonly used for these studies since they are highly sensitive to the antiproliferative properties of IFNs. Not surprisingly, Daudi cells are known to possess a normal RB gene (Resnitsky *et al*., 1992; Kumar and Atlas, 1992). Concurrent with the upregulation of p21 presented here, we have seen similar effects of type I IFNs on p21 in Daudi cells (Subramaniam and Johnson, in press). This may explain why we do not see in DU145 cells the strong G_1 arrest that is seen with Daudi cells upon IFN treatment. In this regard, it would be interesting to determine if a wild type RB gene would increase the sensitivity of DU145 cells to the anticellular effects of IFNs. The inhibition of cyclin E-cdk2 activity by IFNα treatment probably effects other proteins downstream from this activity other than the phosphorylation of pRB. Our findings regarding the effects of IFNα on DU145 cells raise interesting questions about the interplay of various cell cycle regulatory mechanisms, especially in tumor cells that have multiple genetic defects in these regulatory pathways.

Materials and methods

Reagents and cell lines

Purified human IFNα (specific activity 2×10^8 units/ml) was obtained from Biosource International (Camarillo, CA). WISH and DU145 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). Complete media for DU145 cells consisted of Eagles minimal essential medium (EMEM) supplemented with 5% FBS, 200 U/ml penicillin and 200 μg streptomycin. Starve media for cell synchronization contained the above supplemented with 0.5% FBS. Antibodies to cdk2, cyclin E, cyclin D, p21, p27 and p16 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Antiviral assay

IFN activity is expressed in terms of antiviral units/ml as assessed in a standard cytopathic effect assay (Familletti *et al*., 1981). Antiviral activity of human IFNα was determined using the WISH cell line and vesicular stomatitis virus (VSV). One antiviral unit caused a 50% reduction in destruction of the monolayer.

Antiproliferative assays

For colony inhibition studies, anticellular activity was examined using a modification of a colony inhibition assay (Blalock *et al*., 1980). DU145 cells were plated at 600 cells/well in a 24 well plate using complete media with or without IFNα at various concentrations. Plates were incubated 5–6 days to allow for colony formation. Colonies were stained with crystal violet and counted.

IFN inhibition of cell number was determined by using DU145 cells plated in complete media at 1×10^5 cells/well in 6 well plates with or without IFN. At various time points, cells were trypsinized, washed and counted. Cell counts were performed using a hemocytometer and cell viability was assessed by trypan blue dye exclusion.

DNA synthesis assay

DU145 cells were seeded at 2×10^5 cells/well in 6 well plates using starve media for 24 h. Wells were then washed and replaced with complete media alone or media containing IFNα. At 16, 20 and 24 h, cells were harvested and counted as described above. Cells were then reseeded into 96 well plates at 2.5×10^4 cells/well and pulsed with 1 μC [^{3}H]thymidine (specific activity, 21 mCi/mg; 1 Ci = 37 Gbq) (Amersham) for 2 h at 37°C. Cells were then harvested on a model M12 Brandel cell harvester (Gaithersburg, MD) and incorporation of [^{3}H]thymidine was determined using a liquid scintillation counter.

Cell cycle analysis

DU145 cells were synchronized in G_0/G_1 phase (synchronization was assessed by flow cytometry analysis) by culturing in starve media for 24 h. Cells were seeded into 25 cm^2 flasks (Sarstedt, Newton, NC) and treated with media alone or media containing either IFNα. Plates were incubated at 37°C in 5% CO_2 for varying lengths of time. Following incubation, cells were harvested and washed twice using sample buffer (PBS containing 1% glucose) and were then counted. Following centrifugation, cell pellets were resuspended in 100 μl sample buffer and cells were fixed by adding 1 ml cold ethanol (−20°C) dropwise while vortexing. Cells were left in ethanol at 4°C for ⩾24 h. For propidium iodide (Sigma Co, St Louis, MO) staining, the prepared cell samples were washed twice with sample buffer and blotted dry. The cells were then resuspended in 600–800 μl sample buffer containing 50 μg/ml propidium iodide and 100 U/ml RNase A (Sigma Co.) and allowed to stain for up to 1 h at room temperature. Samples were filtered through 44 μm nylon mesh and data was acquired with a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA) using the LYSYS II software system. Analysis of the cell cycle was carried out using CellFIT software.

Immunoprecipitation and immunoblotting

DU145 cells in complete media ($3-6\times10^6$ cells per sample) stimulated with 2500 units/ml IFNα were lysed at 4°C for 20 min in 500 μl of ice-cold lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 20 mM β-glyceryl phosphate, 2 mM Na_3VO_4, 2 mM dithiothreitol, leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (10 μg/ml), benzamidine (5 μg/ml), 1 mM phenylmethanesulfonyl fluoride, 10% (vol/vol) glycerol and 1% (vol/vol) Nonidet P-40. Equal amounts of protein from cell lysates (375–500 μg/500 μl) were subsequently immunoprecipitated with 1 μg of anti-cyclin E or anti-p21. Following Western transfer, membranes were probed with anti-cdk2 or anti-p21 and developed using ECL (Amersham).

In vitro *kinase assays*

Immunoprecipitation of cdk2, cyclin D1 and cyclin E were performed as described above. Immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (50 mM Hepes, pH 7.5, containing 1 mM EGTA, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM β-glyceryl phosphate, 5 μM ATP) and incubated in kinase buffer containing 5 μg histone H1 and 20 μCi of [^{32}P]-γ-ATP (specific activity 6000 Ci/mmol; 1 mCi = 37 Mbq) (Amersham) in a final volume of 30 μl at 30°C for 10 min. Following centrifugation, supernatants (25 μl) were analysed for histone H1 phosphorylation by a filter-binding assay using centrifugal Pierce phosphocellulose units, SpinZyme™ Format purchased from Pierce (Rockford, IL) according to the manufacturer's instructions.

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References

Blalock JE, Georgiades JA, Langford MP and Johnson HM. (1980). *Cell Immunol.*, **49,** 390–394.
Bookstein R, Shew J-Y, Chen P-L, Scully P and Lee W-H. (1990). *Science*, **247,** 712–715.
Burke LC, Bybee A and Thomas NS. (1992). *Oncogene*, **7,** 783–788.
Bybee A and Thomas NS. (1992). *Biochim. Biophys. Acta*, **1137,** 73–76.
Cavenee WK and White RL. (1995). *Sci. Am.*, **March,** 72–79.
Creasy AA, Bartholomew JC and Merigan TC. (1980). *Proc. Natl. Acad. Sci. USA*, **77,** 1471–1475.
Dong J-T, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT and Barrett JC. (1995). *Science*, **268,** 884–886.
Familletti PC, Rubinstein S and Petska S. (1981). *Methods Enzymol.*, **78,** 387–394.
Garnick MB. (1994). *Sci. Am.*, **April,** 72–81.
Gutterman JU. (1994). *Proc. Natl. Acad. Sci. USA*, **91,** 1198–1205.
Isaacs WB, Carter BS and Ewing CM. (1991). *Cancer Res.*, **51,** 4716–4720.
Kumar R and Atlas I. (1992). *Proc. Natl. Acad. Sci. USA*, **89,** 6599–6603.
Nakajima Y, Konno S, Perruccio Y, Chen JM, Wu S, An J, Chiao M, Choudhury M, Mallouh C and Muraki J. (1994). *Biochem. Biophys. Res. Commun.*, **200,** 467–474.
Netto GJ and Humphrey PA. (1994). *Am. J. Clin. Pathol.*, **102,** S57–S64.
Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM and Pagano M. (1995). *Mol. Cell. Biol.*, **15,** 2612–2624.
Parker SL, Tong T, Bolden S and Wingo PA. (1996). *CA Cancer J. Clin.*, **46,** 5–27.
Pines J and Hunter T. (1995). Cycle Control. Hutchinson C andGlover DM (eds). Oxford University Press: New York, pp. 144–176.
Pontzer CH, Bazer FW and Johnson HM. (1991). *Cancer Res.*, **51,** 5304–5307.
Resnitzky D, Tiefenbrun N, Berissi H and Kimchi A. (1992). *Proc. Natl. Acad. Sci. USA*, **89,** 402–406.
Roos G, Leanderson T and Lundgren E. (1984). *Cancer Res.*, **44,** 2358–2362.
Sica G, Fabbroni L, Castagnetta L, Cacciatore M and Pavone-Macaluso M. (1989). *Urol. Res.*, **17,** 111–115.
Subrmaniam PS and Johnson HM. *J Interferon Cytokine Res.*, in press.
Tamm I, Jasny BR and Pfeffer LM. (1987). Mechanisms of Interferon Actions Volume II. Pfeffer LM (ed). CRC Press: Boca Raton, FL, pp. 25–57.
Thomas NS, Burke LC, Bybee A and Linch DC. (1991). *Oncogene*, **6,** 317–322.
Tiefenbrun N, Melamed D, Levy N, Resnitzky D, Hoffmann I, Reed SI and Kimchi A. (1996). *Mol. Cell. Biol.*, **16,** 3934–3944.
Tsai L-H, Lees E, Faha B, Harlow E and Riabowol K. (1993). *Oncogene*, **8,** 1593–1602.