

Molecular Mechanism of Interferon Alfa-Mediated Growth Inhibition in Human Neuroendocrine Tumor Cells

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Background & Aims: Although human neuroendocrine tumors respond to interferon (IFN)- α treatment in vivo, the underlying mechanisms of growth inhibition are poorly understood. To characterize the antiproliferative effects at a molecular level, we explored the growth-regulatory action of IFN- α in the human neuroendocrine tumor cell lines BON and QGP1. **Methods:** IFN- α receptor expression and signal transduction were examined by reverse-transcription polymerase chain reaction, immunoblotting, subcellular fractionation, and transactivation assays. Growth regulation was evaluated by cell numbers, soft agar assays, and cell cycle analysis using flow cytometry. Expression and activity of cell cycle-regulatory molecules were determined by immunoblotting and histone H1-kinase assays. **Results:** Both cell lines expressed IFN- α receptor mRNA transcripts. Ligand binding initiated phosphorylation of Jak kinases and Stat transcription factors, resulting in Stat activation, nuclear translocation, and transcription from an ISRE-reporter construct. Prolonged IFN- α treatment dose-dependently inhibited both anchorage-dependent and -independent growth. Cell cycle analysis of IFN- α -treated, unsynchronized cultures revealed an increased S-phase population, which was further substantiated in G₁ synchronized QGP1 cells. IFN- α -treated cells entered S phase in parallel to control cultures, but their progress into G₂/M phase was delayed. Both cellular cyclin B levels and CDC 2 activity were substantially reduced. The extent and time course of this reduction corresponded to the observed S-phase accumulation. **Conclusions:** IFN- α directly inhibits growth of human neuroendocrine tumor cells by specifically delaying progression through S phase and into G₂/M. These cell cycle changes are associated with inhibition of cyclin B expression, resulting in reduced CDC2 activity.

secrete bioactive molecules, such as regulatory peptides, kinins, and serotonin, which are responsible for the tumor-associated hypersecretion syndrome.² Because more than 80% of malignant GEP tumors present liver metastases at the time of diagnosis, systemic therapy is usually required.¹

Current therapeutic strategies aim to control both hypersecretion-related symptoms and tumor growth.³⁻⁵ Somatostatin and its analogues are effective in suppressing the hypersecretion syndrome, but they are frequently insufficient to control tumor progression.⁶ Therefore, multimodal therapeutic approaches are evaluated that encompass surgical debulking, chemoembolization, chemotherapy, and biotherapy.^{3,5,7} The typically low proliferative fraction of NE tumors limits the efficacy of conventional chemotherapeutic approaches, so that conclusive prolongation of survival remains to be demonstrated.^{8,9} In view of the potential side effects of chemotherapeutic regimes, biotherapeutic approaches using long-term treatment with interferon (IFN)- α alone or in combination with somatostatin analogues have widely replaced conventional chemotherapeutic strategies over the past decade.^{3-5,7,10}

Patients with advanced GEP NE tumor disease have received IFN- α in the context of clinical trials; a significant fraction of tumors responded to IFN- α treatment, as determined by biochemical markers such as 5-hydroxyindolacetic acid excretion or chromogranin A serum levels.^{5,7,11} IFN- α -induced reduction of such biochemical markers was reflected by a marked relief of hypersecretion-associated symptoms in functionally active tumors. Several of these studies also reported an

Abbreviations used in this paper: DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; FCS, fetal calf serum; GED, gastroenteropancreatic; IFN, interferon; ISRE, interferon-stimulated response element; PKR, RNA-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Neuroendocrine (NE) gastroenteropancreatic (GEP) tumors constitute a biologically heterogeneous group of rare, predominantly slow-progressing but mostly malignant neoplasms.¹ They frequently synthesize and

IFN- α -mediated inhibition of tumor progression resulting in stable disease or, in some cases, partial remission with reduction of tumor burden.^{11,12} It is of particular clinical relevance that inclusion of IFN- α in the therapeutic regimen enabled suppression of disease progression in a significant fraction of tumors resistant to monotherapy with somatostatin analogues.⁵ Nonetheless, a significant fraction of endocrine GEP tumors fail to respond to the growth-inhibitory effects of IFN- α . The clinical benefit of the specific antiproliferative actions of IFN- α has therefore remained controversial.

Multiple mechanisms have been proposed to contribute to overall clinical outcome of IFN- α treatment, including direct antiproliferative actions of IFN- α on the tumor cells and indirect mechanisms, such as inhibition of angiogenesis, induction of tumor mesenchyme, and immunomodulation via up-regulation of major histocompatibility class I antigens (reviewed by Grander et al.¹³). Resistance of individual NE tumors to the growth-regulatory effects of IFN- α may result from defective signal transduction or tumor specific phenotypic alterations in growth-regulatory pathways. Because the responsiveness of the secretory pathway is frequently maintained in functionally active GEP tumors that are refractory to the growth-inhibitory actions of IFN- α ,¹⁰⁻¹² the latter mechanism seems to be of major biological relevance in endocrine GEP tumors. Consequently, identification of the specific growth-relevant cellular targets of IFN- α and understanding of their function are needed to maximally exploit the potential of IFN- α in the treatment of NE tumor disease.

Much progress has been made in elucidating the signal transduction pathways activated by type I IFNs.^{14,15} Ligand binding to specific receptors at the plasma membrane results in dimerization of receptor subunits and thereby initiates the rapid autophosphorylation of receptor-associated Janus tyrosine kinases (JAKs) Jak1 and Jak2. The activated JAKs in turn tyrosine-phosphorylate and activate latent cytosolic members of the Stat transcription factor family, named for their dual function as signal transducers and activators of transcription. Activated Stat1 and Stat2 transcription factors then associate with a 48-kilodalton protein into multimeric complexes termed IFN-stimulated gene factor 3 (ISGF3) that translocate into the nucleus where they induce changes in gene expression, which ultimately result in the biological effects of IFN- α treatment.

In contrast to these well-established and conserved signal transduction events, the molecular basis of the antiproliferative effects of IFN- α appears to be highly cell type specific and has not yet been delineated in GEP NE tumor cells. The most comprehensive analysis of IFN- α -

induced growth regulation used Daudi Burkitt lymphoma cells and identified multiple signaling pathways that apparently function in parallel to block G₁-S progression. Two major independent and complementary effector pathways have emerged from these studies. The first is accumulation of the hypophosphorylated, growth-restrictive form of the retinoblastoma-associated tumor-suppressor protein Rb.¹⁶⁻²¹ This functional activation of Rb is attributed to down-regulation of G₁ cyclins and CDC25 phosphatases in conjunction with induction of cyclin-dependent kinase inhibitors (CKIs) and total Rb content. The second pathway is induction of the double-stranded RNA-activated protein kinase (p68 PKR) initiating down-regulation of cellular c-myc levels.^{22,23} However, alternative mechanisms may prevail in NE tumors that have been reported to exhibit functional inactivation of Rb²⁴ and overexpression of c-myc.²⁵ To specifically evaluate the direct cell cycle-regulatory effects of IFN- α on GEP NE tumor cells, we analyzed IFN- α -dependent signal transduction and growth regulation in an in vitro model of human GEP NE tumor disease.

Materials and Methods

Materials

QGP1 cells were kindly provided by K. Mölling (Zürich, Switzerland); BON cells were a generous gift from C. M. Townsend (Galveston, TX). Dulbecco's modified Eagle medium (DMEM), RPMI 1640 medium, and phosphate-buffered saline (PBS) were supplied by GIBCO BRL (Berlin, Germany). Fetal calf serum (FCS), trypsin/EDTA, penicillin, and streptomycin were from Biochrom (Berlin, Germany). The antibodies were purchased from the following manufacturers: Santa Cruz Biochemicals (Santa Cruz, CA; CDK2, Jak1, Tyk2, Stat1, Stat2); Transduction Laboratories (Lexington, KY; p27^{kip1}); Pharmingen (San Diego, CA; cyclins E, A, and B); Calbiochem-Novabiochem GmbH (Bad Soden, Germany; p21^{cip1}); and Dianova GmbH (Hamburg, Germany; all secondary antibodies). [γ -³²P]Adenosine triphosphate (ATP) and enhanced chemiluminescence Western blotting detection reagents were obtained from Amersham (Braunschweig, Germany), and calf histone H1, deoxyribonuclease, and ATP from Boehringer Mannheim (Mannheim, Germany). Polymerase chain reaction (PCR) reagents were obtained from Promega (Heidelberg, Germany), and Western blot supplies from Bio-Rad Laboratories GmbH (München, Germany). IFN- α 2b (Roferon) was kindly provided by Hofmann-LaRoche (Basel, Switzerland). Protein A-Sepharose beads and all other reagents were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

Cell Lines and Tissue Culture

The human NE pancreatic tumor cell lines QGP1 and BON were grown as subconfluent monolayer cultures in RPMI

1640 and DMEM, respectively, supplemented with 10% (vol/vol) FCS, 100 U/mL penicillin, and 100 μ g/mL streptomycin, and kept in 95% air and 5% CO₂ at 37°C. Experiments were routinely performed in the log phase of growth after cells had been plated for 16–24 hours. For experiments on synchronized cultures, QGP1 cells were serum starved for 36 hours, which resulted in G₁-phase accumulation of approximately 70% of the cells, and were then restimulated by addition of 10% FCS.

Cell Growth Assays

Effects of IFN- α on proliferation of NE tumor cells were evaluated by determination of cell numbers. Cells were plated at 50,000 cells/well (BON) or 25,000 cells/well (QGP1) in 12-well tissue culture plates, and cells were manually counted using a hemacytometer at indicated time points. Viability of cells was confirmed by trypan blue exclusion and was routinely >95%. To determine effects of IFN- α on anchorage-independent growth of NE tumor cells, colony formation in agar suspension was evaluated. Briefly, 3×10^4 cells were resuspended in 300 μ L of culture medium and added to a mixture of 2.7 mL Hyclone FCS, 0.8 mL Iscove's modified DMEM, 3.6 mL of 2.1% (wt/vol) methylcellulose in Iscove's, 1.6 mL agar solution (10 mL 3% agar and 20 mL DMEM), and 0.06 mL β -mercaptoethanol to obtain an agar suspension. Aliquots (1 mL, 3000 cells) of this suspension were then dispensed in 35-mm dishes containing the indicated concentrations of IFN- α or vehicle. Colony formation was assessed under an inverted microscope by manual counting after a 10-day incubation period. A threshold of 20 cells was arbitrarily set to score cell accumulations as colonies.

Flow-Cytometric Analysis

For cell cycle analysis, approximately 10^6 cells were harvested by gentle trypsinization (0.25%), carefully resuspended in PBS, and fixed in ethanol (70%) for 30 minutes at -20°C. After brief centrifugation, cells were washed once with PBS and incubated for 30 minutes at room temperature in PBS containing 100 μ g/mL ribonuclease A, 0.1% Triton X-100, 1 μ mol/L EDTA, and 1.5 μ g/mL propidium iodide. Cell cycle analysis was carried out on a FACScan using Modfit software (Becton Dickinson, Heidelberg, Germany).

Western Blotting and Subcellular Fractionation

For analysis of cell cycle proteins, subconfluent cells were treated as indicated, rinsed twice with ice-cold PBS containing 1 mmol/L sodium orthovanadate (Na₃VO₄), and extracted in ice-cold lysis buffer (20 mmol/L Tris [pH 7.8], 150 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L β -glycerophosphate, 0.5% Nonidet P-40, 1% glycerine, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol [DTT], 2 μ mol/L phenylmethylsulfonyl fluoride [PMSF], 10 μ g/mL aprotinin, and 2 μ mol/L leupeptin). Extracts were boiled in Laemmli's sample buffer, and aliquots were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

(SDS-PAGE), electroblotted to polyvinyl difluoride membranes (New England Nuclear, Köln, Germany), and blocked in PBS/0.1% Tween (PBST) containing 5% nonfat dry milk (cyclins, E, A, B, p21^{cip1}, p27^{kip1}, Rb, p16, Jak1, Tyk2, Stat1, Stat2) or 5% bovine serum albumin (CDK2, CDC2) for 2 hours at room temperature. Incubation with primary antibodies was carried out overnight at 4°C and was followed by 3 washes in PBST at room temperature. After incubation with appropriate horseradish peroxidase–conjugated secondary antibodies, membranes were washed 3 times for 15 minutes in PBST, and bands were visualized by enhanced chemiluminescence following the manufacturer's recommendations. Results were quantitated by laser densitometry using the Scanpak 2 software (Biomtra, Göttingen, Germany).

For Stat translocation studies, subcellular fractions of QGP cells were prepared before immunoblotting according to the procedure described by Simboli-Campbell.²⁶ After hypotonic lysis (1 mmol/L NaHCO₃, 5 mmol/L MgCl₂, 0.1 mmol/L PMSF, and 20 μ g/mL leupeptin), extracts were adjusted to 50 mmol/L Tris-HCl (pH 7.5) plus 50 mmol/L EGTA and nuclei were isolated by brief centrifugation at 500g. Nuclei were further purified by saccharose gradient centrifugation (45% [wt/vol] saccharose, 30 minutes, 1660g) and then lysed in buffer A containing 50 mmol/L Tris-HCl (pH 7.5), 0.5 mmol/L EGTA, 0.5 mmol/L EDTA, 1% β -mercaptoethanol, 1 mmol/L PMSF, 20 μ g/mL leupeptin, and 1% SDS by brief sonication. Aliquots of the nuclear and the cytosolic fractions were then processed as described above.

Immunoprecipitation and Histone H1–Kinase Assays

To determine tyrosine phosphorylation of Jak kinases and Stat proteins, subconfluent cells were treated as indicated, then washed twice in ice-cold PBS and lysed by brief sonication in IP buffer (20 mmol/L Tris [pH 7.8], 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% Nonidet P-40, 10 mmol/L NaF, 10 mmol/L sodium orthovanadate, 2 mmol/L PMSF, and 10 μ g/mL each of leupeptin, pepstatin, and aprotinin). The lysates were precleared for 1 hour by incubation with protein A–Sepharose beads. Immune complexes were collected on protein A–Sepharose beads that had been coated with saturating amounts of primary antibody. Beads were subsequently washed 4 times with ice-cold IP buffer, boiled in Laemmli's sample buffer, separated by SDS-PAGE, and transferred to polyvinyl difluoride membranes. Nonspecific binding was blocked with 10 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, and 0.05% Tween 20 containing bovine serum albumin (1%) for 2 hours at room temperature. The membrane was then incubated with α -phosphotyrosine antibody (1 μ g/mL) for 2 hours at room temperature and further processed as described for Western blots. To control that equivalent amounts of immunocomplexes were analyzed, membranes were reprobed with the antibodies used for immunoprecipitation. For repeated use, membranes were stripped by incubating the membrane in a solution of 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, and 100 mmol/L 2-mercaptoethanol for 30 minutes at 55°C followed by a

washing in PBST for 2 hours, changing the buffer every 30 minutes.

To examine the composition and activity of cdk complexes, immunoprecipitations were performed. For immunoprecipitation of CDK2 or CDC2, cells were lysed by mild sonication in ice-cold ELB buffer (50 mmol/L HEPES [pH 7.5], 250 mmol/L NaCl, 5 mmol/L EDTA, 0.1% Nonidet P-40, 1 mmol/L DTT, 1 mmol/L NaF, 0.1 mmol/L Na_3VO_4 , 2 $\mu\text{g/mL}$ aprotinin, 5 $\mu\text{g/mL}$ leupeptin, and 0.1 mmol/L PMSF) followed by a 30-minute incubation on ice with occasional vortexing. After brief centrifugation (10 minutes, 4°C, 13,000g), lysates (500 $\mu\text{g/sample}$) were precleared for 1 hour by incubation with protein A–Sepharose beads. Immunocomplexes were then precipitated by addition of protein A–Sepharose beads precoated with saturating amounts of CDK2 or CDC2 antibody (10 μg). Samples were incubated with gentle agitation at 4°C for 4 hours. Immunocomplexes were subsequently washed 4 times in ice-cold ELB buffer and twice in 50 mmol/L HEPES (pH 7.5) containing 1 mmol/L DTT. The kinase reaction was then started by addition of 30 μL kinase buffer containing 50 mmol/L HEPES (pH 7.5), 1 mmol/L DTT, 10 mmol/L MgCl_2 , 1 μg calf histone H1 per sample, 50 $\mu\text{mol/L}$ ATP, and 5 μCi [$\gamma\text{-}^{32}\text{P}$]ATP per sample. The kinase reactions were allowed to proceed for 5 minutes (CDK2) and 30 minutes (CDC2) and were then terminated by boiling the samples in Laemmli's buffer. The whole reaction was subjected to 10% SDS-PAGE, and kinase activities were determined by autoradiography of the dried gels.

Transactivation Studies

To determine transactivation after IFN- α stimulation in BON NE tumor cells, transient transfection assays with an IFN-stimulated response element (ISRE)-luciferase reporter gene construct were performed. The ISRE-luc construct, containing residues -206/-86 of the human 2',5'-oligoA synthetase enhancer, and a mutated variant were a generous gift from Shoumo Bhattacharya²⁷ and were subcloned into pTGL2 promoter vector (Promega, Madison, WI). BON cells (5×10^5 cells/well) were plated in 6-well tissue culture dishes, and transfections were carried out by calcium phosphate precipitation technique using a DNA transfection kit (5 Prime-3 Prime; Boulder, CO), exactly as described previously.²⁸ Transfected cells were allowed to recover for 16 hours and then stimulated for 24 hours with the indicated IFN- α concentrations. After lysis of cells, the luciferase activity was determined using luciferin, ATP, and coenzyme A (Promega) exactly as reported previously.²⁸

RNA Preparation and RT-PCR

To confirm the presence of IFN- α receptor messenger RNA (mRNA) transcripts in QGP1 and BON cells, IFN- α receptor mRNA was amplified by RT-PCR. Total RNA from NE tumor cells was prepared using RNeasy R reagent (WAK Chemie, Bad Soden, Germany) according to the manufacturer's instructions. RNA was submitted to deoxyribonuclease digestion, and aliquots of 1 μg were used for reverse transcription

using Moloney murine leukemia virus reverse transcriptase. The reaction was diluted 1:25 for use in the subsequent PCR reaction with sequence-specific primers to a 639 base pair (bp)-spanning region of the human IFN- α receptor complementary DNA (cDNA) (5'-AGC GAT GAG TCT GTC GGG; 3'-GGC GTG GAG CCA CTG AAC). Amplification conditions were exactly as previously described.²⁹

Results

Human NE Tumor Cells Express Functional IFN- α Receptors

As a first step to establish the human endocrine GEP tumor cell lines QGP1 and BON as a suitable *in vitro* model to investigate IFN- α actions, we determined expression of IFN- α receptors at the mRNA level by RT-PCR analysis. Using sequence-specific primers against the IFN- α receptor cDNA, amplicates of the predicted size (639 bp) were detected in both NE cell lines as well as in Capan1 exocrine pancreatic cancer cells, which were included as positive control²⁹ (Figure 1).

To confirm that the mRNA transcripts corresponded to functionally active receptors, ligand-initiated signal transduction was examined next. IFN- α receptors consist of 2 chains that associate with Jak1 and Tyk2 kinases. Upon IFN- α binding to the receptor, Jak1 and Tyk2 kinases approach each other and become activated by phosphorylation on tyrosine residues. To investigate IFN- α -dependent phosphorylation of JAKs in NE tumor cell lines, Jak1 (Figure 2, upper panel) and Tyk2 (Figure 2, lower panel) were immunoprecipitated from whole-cell lysates and subsequently analyzed for tyrosine phosphorylation by immunoblotting. After IFN- α stimulation, Jak1 immunoprecipitates from QGP1 cells readily increased their complement of phosphotyrosine, starting as early as 1 minute and reaching a maximum at 15 minutes. In contrast to the results obtained in QGP1

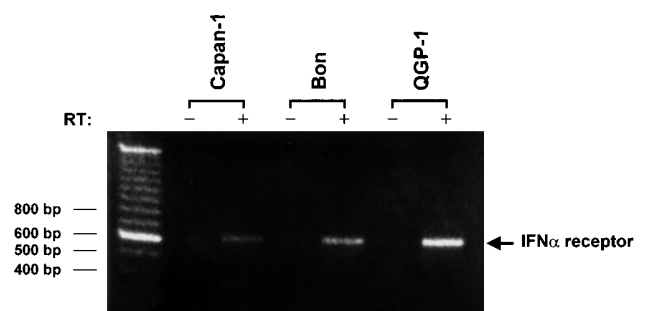


Figure 1. NE tumor cells express IFN- α receptor mRNA. Total RNA was extracted from QGP1, BON, and Capan1 cell lines, reverse-transcribed, and amplified by PCR using primers directed against the type I IFN receptor α chain. For size determination of the obtained PCR fragment, a 100-bp DNA standard was used (*lane 1*). Alternating sample lanes represent reactions with (+) or without (-) addition of reverse transcriptase.

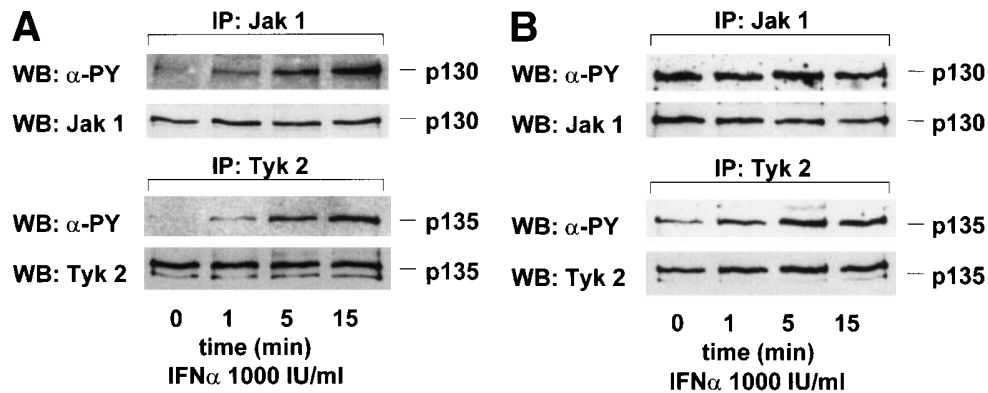


Figure 2. IFN- α treatment initiates activation of receptor-associated kinases in human NE tumor cells. Immunoblots of Jak1 and Tyk2 immunoprecipitates analyzing IFN- α -induced changes in the phosphotyrosine content of IFN- α receptor-associated kinases in (A) QGP1 and (B) BON cells. Cells were incubated with 1000 IU/mL IFN- α for the indicated periods. Blots were subsequently stripped and reprobed with antibodies against Jak1 or Tyk2 to confirm that equal amounts of immunocomplexes were evaluated. Because Jak1 phosphotyrosine signals were notably weaker in BON cells than in QGP1 cells, the autoradiography had to be exposed for 30 minutes compared with 1–2-minute exposures required in QGP1 cells.

cells, little increase in Jak1 tyrosine phosphorylation was observed in IFN- α -treated BON cells (Figure 2B), although weak tyrosine phosphorylation was consistently observed under control conditions. However, both QGP1 and BON cells demonstrated a distinct, time-dependent increase in Tyk2 tyrosine phosphorylation, indicative of Tyk2 activation at 1, 5, and 15 minutes after IFN- α stimulation. Subsequent immunoblotting of the stripped phosphotyrosine blots with antibodies against Jak1 (Figure 2, upper panel, second row) and Tyk2 (Figure 2, lower panel, second row) confirmed that equal amounts of immunocomplexes had been analyzed in IFN- α -treated cells and control cultures.

In response to ligand-dependent IFN- α receptor activation, latent cytoplasmic Stat1 and Stat2 transcription factors are recruited to specific binding sites on the cytoplasmic portion of the receptor, where Jak kinases then activate these Stat proteins via phosphorylation on tyrosine residues. Tyrosine phosphorylation of Stat proteins was therefore examined to confirm IFN- α -induced

activation of downstream effector molecules. In QGP1 (Figure 3A) and BON cells (Figure 3B), a pronounced, time-dependent increase in Stat 1 tyrosine phosphorylation (Figure 3, upper panel) was evident. Enhanced phosphorylation was detected on both the 91- and 84-kilodalton splice variants, with stronger signals for the 91-kilodalton form most likely reflecting its higher abundance in human NE cells (Figure 3, upper panel, second row). Similarly, phosphorylation of Stat2 was enhanced (Figure 3, lower panel), although the increase was moderate compared with the changes observed for Stat1. Comparison of the time courses of Stat activation in both cell lines revealed slightly different kinetics. Phosphorylation of Stat proteins in QGP1 was transient, with maximal effects occurring at 15 minutes of IFN- α stimulation for Stat1 and at 5 minutes for Stat2. In BON cells, phosphorylation of Stat1 and Stat2 was first evident at 5 minutes and thereafter persisted to 60 minutes with Stat1 phosphotyrosine levels showing a continuous increase over this period of time.

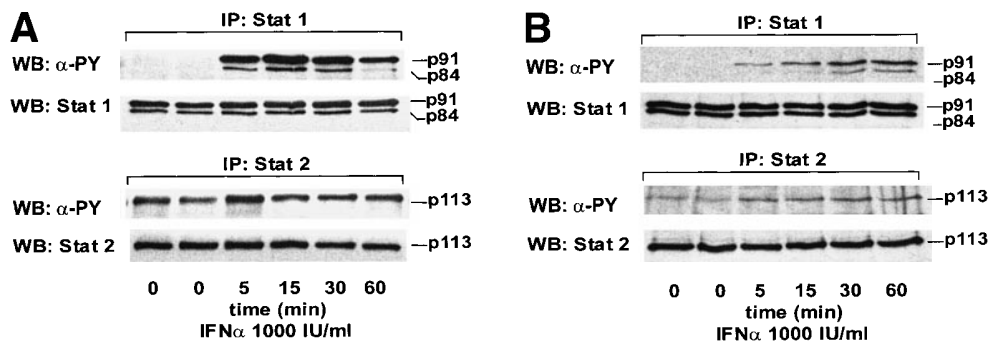


Figure 3. IFN- α time-dependently induces tyrosine phosphorylation of Stat1 and Stat2 in NE tumor cells. Both tyrosine phosphorylated (upper row) and total (lower row) Stat complement of Stat1 (upper panel) and Stat2 immunoprecipitates (lower panel) from IFN- α -treated cells were evaluated by immunoblotting. Cells were treated with 1000 IU/mL IFN- α for the indicated intervals. p91 and p84 indicate Stat 1 α - and β -splice variants, respectively, based on their molecular weight. Data are from representative experiments in (A) QGP1 and (B) BON cells.

After activation of latent Stat transcription factors by phosphorylation, Stat1 and Stat2 form heterodimers that translocate into the nucleus, where they initiate transcription from ISREs. To directly determine IFN- α -dependent transactivation of ISREs in NE cells, reporter gene studies were performed. In transient transfection experiments using BON cells, IFN- α dose-dependently stimulated transcription from an ISRE-luciferase reporter construct, but not from a mutant ISRE (Figure 4A), indicating that IFN- α -induced Stat signaling results in specific transactivation of IFN-regulated genes in this cell line. Because we repeatedly failed to achieve acceptable expression levels of the reporter construct in transfected QGP1 cells, nuclear translocation was determined as a substitute for reporter gene studies. IFN- α stimulation led to a time-dependent increase of Stat1 and Stat2 proteins in nuclear extracts of QGP1 cells (Figure 4B), documenting the translocation of activated Stat proteins in response to IFN- α .

IFN- α Inhibits Growth of Human NE Tumor Cells

Having established an intact IFN- α -signaling machinery in both cell lines, we next addressed the question of whether IFN- α exhibits a direct antiproliferative action on QGP1 and BON cells. Growth curves in the presence or absence of IFN- α were obtained over a period of 6 days (Figure 5A). IFN- α treatment (1000 IU/mL) resulted in a significant inhibition of proliferation in QGP1 (Figure 5A) and in BON cells (Figure 5B). The growth-inhibitory effects of IFN- α were concentration dependent (Figure 5C and D) with half maximal effects on proliferation occurring at less than 100 IU/mL in QGP1 and at approximately 500 IU/mL in BON cells, which is within the therapeutically achievable range of plasma levels in humans.

Growth of malignant tumors in vivo is influenced by unique properties of transformed cells, which are more closely reflected by anchorage-independent growth assays than by substrate-dependent proliferation assays. To establish that IFN- α is capable of directly regulating anchorage-independent growth of NE tumor cells, colony formation of QGP1 and BON cells in agar suspension was evaluated. IFN- α treatment profoundly inhibited substrate-independent growth of QGP1 cells in a dose-dependent manner (Figure 6A) with a maximal decrease in colony number to $15\% \pm 5\%$ of control achieved with 1000 IU/mL IFN- α . Similarly, substrate-independent growth of BON cells was impaired (Figure 6B), although the maximal reduction to 77% of control was only moderate and did not reach statistical significance. Thus, both substrate-dependent and substrate-independent growth of NE tumor cells were directly inhibited by IFN- α .

IFN- α -Treated NE Tumor Cells Accumulate in the S Phase of the Cell Cycle

Depending on the cell model investigated, various mechanisms responsible for the growth-inhibitory effects of IFN- α have been suggested, most of which involve modulation of Rb. To test whether Rb might also be involved in IFN- α -mediated growth inhibition of NE tumor cells, immunoblotting experiments were performed to analyze expression and phosphorylation status of Rb in QGP1 and BON cells (Figure 7). These blots revealed a striking difference in the Rb content of the 2 cell lines. Bands representing the hypophosphorylated and hyperphosphorylated forms of Rb were easily detected both in BON and exocrine Capan1 cells, but no signal could be visualized in QGP1 cells when equivalent amounts of protein were examined. Control blots using an antibody against p16^{ink4a} showed a complementary

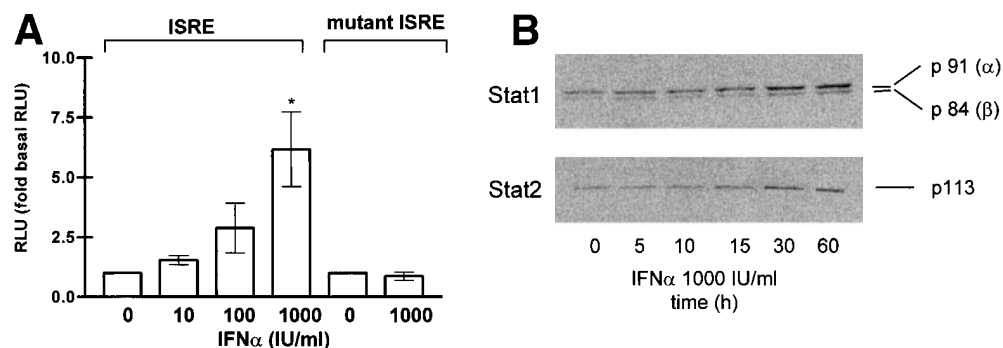


Figure 4. IFN- α stimulation results in transactivation of an ISRE-driven reporter construct. (A) Dose-dependent increase of relative luciferase activity (RLU) in IFN- α -treated BON cells transiently transfected with pGL2-ISRE-luc, but not mutant pGL2- Δ ISRE-luc. Data represent means $\pm$ SEM from at least 3 separate experiments conducted in triplicate. (B) Immunoblot analysis of Stat1 and Stat2 in nuclear extracts of QGP1 cells, confirming the nuclear translocation of Stat transcription factors in cells stimulated with 1000 IU/mL IFN- α for the indicated times.

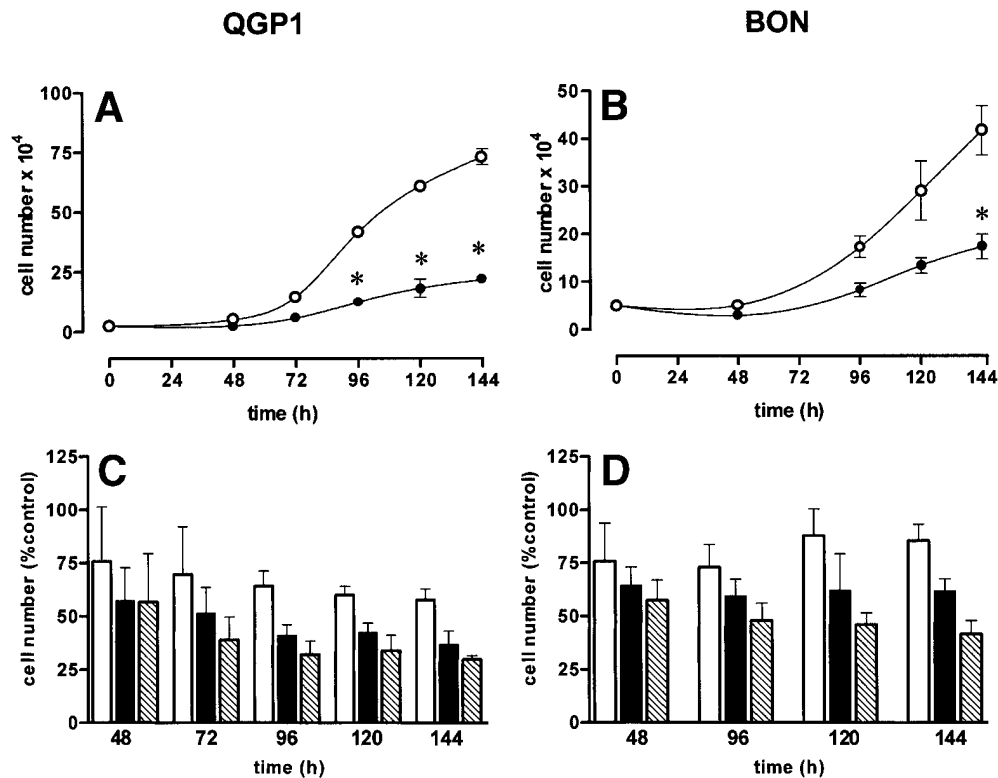


Figure 5. IFN- α inhibits growth of NE tumor cells in a time- and dose-dependent manner. Subconfluent cells were treated with (A and B) 1000 IU/mL IFN- α (●) or vehicle (○) for the indicated time periods or with (C and D) 100 (□), 500 (■), and 1000 (▨) IU/mL for the indicated times. Viable cells were then manually counted in a hemacytometer. Data represent means $\pm$ SEM from at least 3–4 separate experiments, each conducted in triplicate. * $P \leq 0.05$ compared with vehicle-treated controls.

expression pattern, as previously described for a variety of cells.³⁰ Neither the expression level of p16^{ink4a} nor the relative abundance of the hypophosphorylated form of Rb were up-regulated by IFN- α treatment, whereas overall pRb content was even moderately reduced at 48 hours (Figure 7), indicating that the growth inhibition observed in NE tumor cells did not require activation of Rb.

To identify potential mechanisms responsible for the growth-regulatory effects of IFN- α in NE cells, IFN- α -induced cell cycle redistribution was determined by flow

cytometry (Figure 8). In these experiments on unsynchronized cells, IFN- α treatment increased the fraction of cells in the S phase of the cell cycle, whereas the proportion of cells in the G₁ phase decreased (Figure 8A). Both QGP1 and BON cell lines displayed a comparable redistribution pattern (Figure 8B), again supporting the notion that IFN- α elicits Rb-independent cell cycle-specific effects in NE cells. Of note, no population with

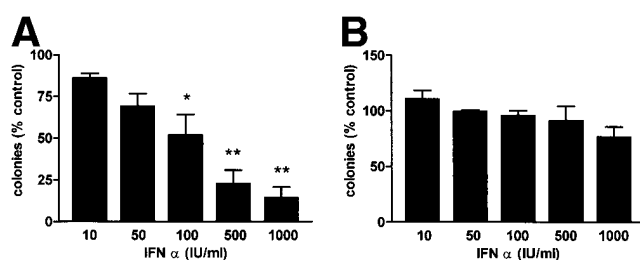


Figure 6. IFN- α inhibits anchorage-independent growth of NE tumor cells. A single cell agar suspension of (A) QGP1 or (B) BON cells was incubated with the indicated doses of IFN- α for 10 days, and colony formation was evaluated. Data represent means $\pm$ SEM from at least 3 separate experiments, each conducted in triplicate. * $P \leq 0.05$ and ** $P \leq 0.01$ compared with vehicle-treated controls.

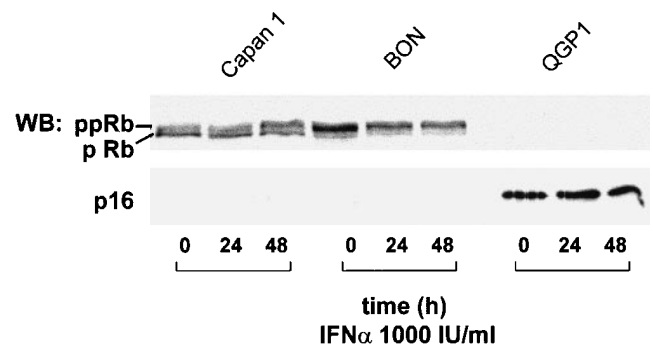


Figure 7. IFN- α treatment does not affect Rb phosphorylation in NE tumor cells. (A) Immunoblot analysis of Rb expression and phosphorylation status in control and IFN- α -stimulated NE tumor cells as well as Capan1 exocrine pancreatic cancer cells. In each lane, 15 μ g of whole cell lysates was separated by 7.5% SDS-PAGE. (B) Aliquots of the same lysates immunoblotted for expression of the CKI p16^{ink4a}.

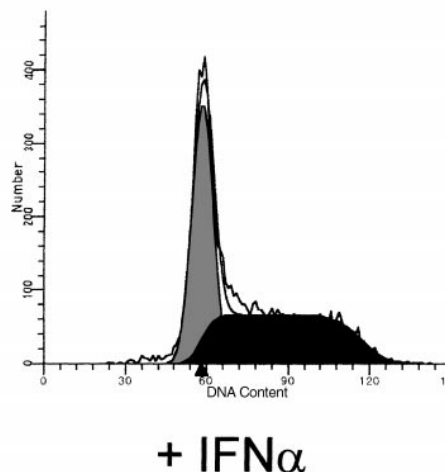
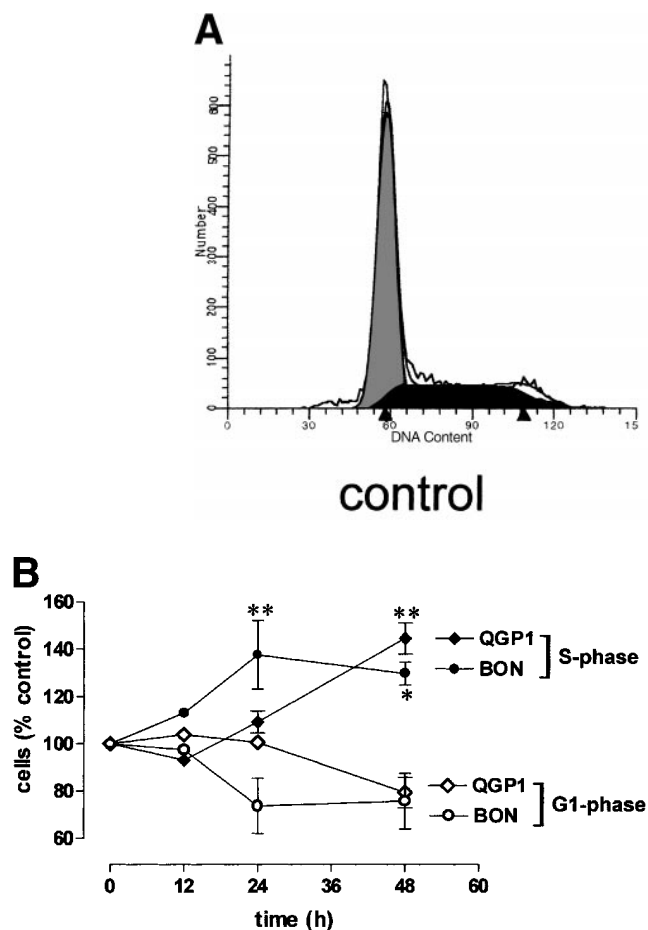


Figure 8. IFN- α -treated NE tumor cells accumulate in the S phase of the cell cycle. (A) Representative fluorescence-activated cell sorter analysis showing changes in cell cycle distribution of QGP1 cells treated with 1000 IU/mL IFN- α for 48 hours. (B) Summary of IFN- α -induced cell cycle redistribution in QGP1 and BON cells. Data are expressed as mean $\pm$ SEM of the percentage of cells in the S phase (closed symbols) and the G₁ phase (open symbols) compared with untreated control cultures, determined in 3 independent experiments. * $P \leq 0.05$ and ** $P \leq 0.01$ compared with control.

subdiploid DNA content was observed in these experiments, indicating that no relevant induction of apoptotic cell death had occurred. Analysis of the kinetics of IFN- α -induced cell cycle effects revealed first S-phase accumulation at 24 hours of treatment (Figure 8B) and a further, significant increase of the S-phase fraction up to 48 hours (Figure 8A and B).

Because there was no qualitative difference between the 2 NE cell lines, QGP1 cells were used in all subsequent experiments as a representative model to examine cell cycle-regulatory events in more detail. Unaltered passage of IFN- α -treated cells along the G₁ restriction point was supported by unchanged cellular cyclin E levels and CDK2 activity (Figure 9A), as determined by immunoblotting and H1 kinase assays, respectively. Also, cellular levels of the stoichiometric cyclin-dependent kinase inhibitors p21^{cip1} and p27^{kip1}, previously reported to be regulated by IFN- α , were not significantly increased in IFN- α -treated NE tumor cells (data not shown). In contrast, both S phase-associated cyclins, cyclin A and cyclin B, showed a subtle decrease at 12 hours, followed by a transient increase at 18 and 24

hours, and finally returned to subbasal levels at 48 hours (Figure 9B).

Although these minor changes in S phase-associated cyclins did not result in a functionally relevant regulation in CDC2 kinase activity within the first 24 hours (Figure 9B, lower panel), the pattern of regulation strongly suggested the possibility of transient IFN- α effects resulting in a partial synchronization of the treated cell cultures. Because such a partial synchronization precludes definitive interpretation of the observed cell cycle redistribution, cell cycle effects of IFN- α were further analyzed in synchronized QGP1 cells (Figure 10A). By serum starvation over a period of 36 hours, reasonable synchronization was achieved with approximately 70% of cells in the G₁ phase of the cell cycle. QGP1 cells were then stimulated to resume progression through the cell cycle by addition of fetal calf serum, and treatment groups received 1000 IU/mL IFN- α at the time of serum stimulation. Cell cycle analyses of IFN-treated cells and time-matched controls were compared during the first cycle until control cells reentered G₁ and synchronization was lost. Control cultures proceeded through S and G₂/M

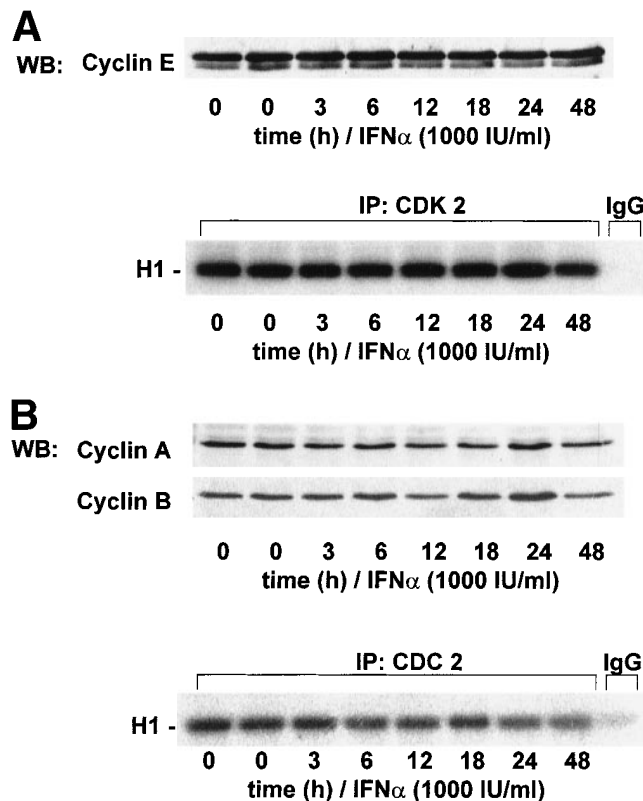


Figure 9. IFN- α treatment does not impair CDK2 activity in randomly cycling QGP1 cells. (A) Effects of IFN- α on expression of cyclin E and on CDK2 activity as determined by immunoblotting and histone H1-kinase assays, respectively. (B) Immunoblot analysis to determine IFN- α -dependent regulation of S-G₂/M phase cyclin expression (cyclin A and B, upper panel) and determination of histone H1-kinase activity of CDC2 complexes prepared from randomly cycling cells (lower panel). Immunoblots and kinase assays are representative of at least 2–3 experiments.

phases within 15 hours and 18–21 hours of serum addition, respectively (Figure 10A, upper panel), and a substantial portion of cells had reentered G₁ by 24 hours. Entry of IFN- α -treated cells into S phase paralleled that of control cultures. However, in contrast to control cells, the treated cells failed to enter the G₂/M phase by 18 hours, indicating impaired progression through S phase. The transition through S phase was delayed rather than blocked, because a small portion of treated cells could be observed to enter G₂/M by 21 hours and a substantial G₂/M peak was evident at 24 hours (Figure 10A, lower panel). A quantitative evaluation of cell cycle progression in IFN-treated compared with control cells from various experiments confirmed the transient S-phase accumulation followed by progression into G₂/M between 21 and 24 hours of serum stimulation (Figure 10B).

Because the experiments on synchronized cells confirmed that G₁/S transition of NE tumor cells was not affected by IFN- α , a more detailed analysis of cell

cycle-regulatory events was restricted to cdk complexes acting at the S and G₂/M transition. Again, cyclin A and cyclin B expression in synchronized cells were examined by immunoblotting. As expected, cyclin B was induced as control cells progressed through the cell cycle. In contrast, cell cycle-dependent induction of cyclin B was clearly delayed and substantially blunted in the IFN- α -treated cultures (Figure 11A). Because cyclin B induction is a prerequisite for CDC2 activation during G₂/M progression, impairment of cyclin B induction should result in reduced CDC2 activity. In agreement with this prediction, the analysis of CDC2 kinase activity revealed a distinct inhibition at 15, 18, and 21 hours of IFN- α treatment (Figure 11B). Alignment of cyclin B expression levels and CDC2 kinase activity in IFN- α -treated cells (Figure 11C) showed a close correlation in both extent and time course of regulation. Thus, the IFN- α -induced reduction of CDC2 activity most likely resulted from inhibition of cyclin B induction. Moreover, the kinetics of CDC2 inhibition exactly corresponded to the transient S-phase accumulation noted in the cell cycle analyses (Figure 10B).

Discussion

In the clinical management of NE GEP tumor disease, IFN- α treatment will stabilize tumor progression in a subgroup of patients. However, the mechanisms that operate to restrain tumor cell proliferation are poorly understood. The transforming events that give rise to sporadic NE tumors are not yet well defined. From limited reports in the literature it appears that common events during carcinogenesis, such as loss of p53 function or K-ras mutations, are notably absent in most endocrine GEP tumors.²⁵ Also, specific Menin defects, as observed in the familiar multiple endocrine neoplasia syndrome, are found only in a subset of sporadic endocrine GEP tumors.³¹ The current study was conducted to delineate direct growth-regulatory mechanisms of IFN- α in the specific phenotypic context of NE tumor cells at the cell cycle level and thereby elucidate the molecular basis for empirically derived therapeutic benefit.

The data establish that 2 representative NE tumor cell lines, QGP1 and BON, express functionally competent IFN- α receptors that initiate activation of the Jak-Stat signaling pathway. This is consistent with results from a previous study showing enhanced DNA binding of Stat complexes in IFN- α -stimulated BON cells.³² That study characterized Stat3 and Stat5 as the predominantly activated isoforms, but their functional relevance in the context of growth-regulatory actions of IFN- α has not

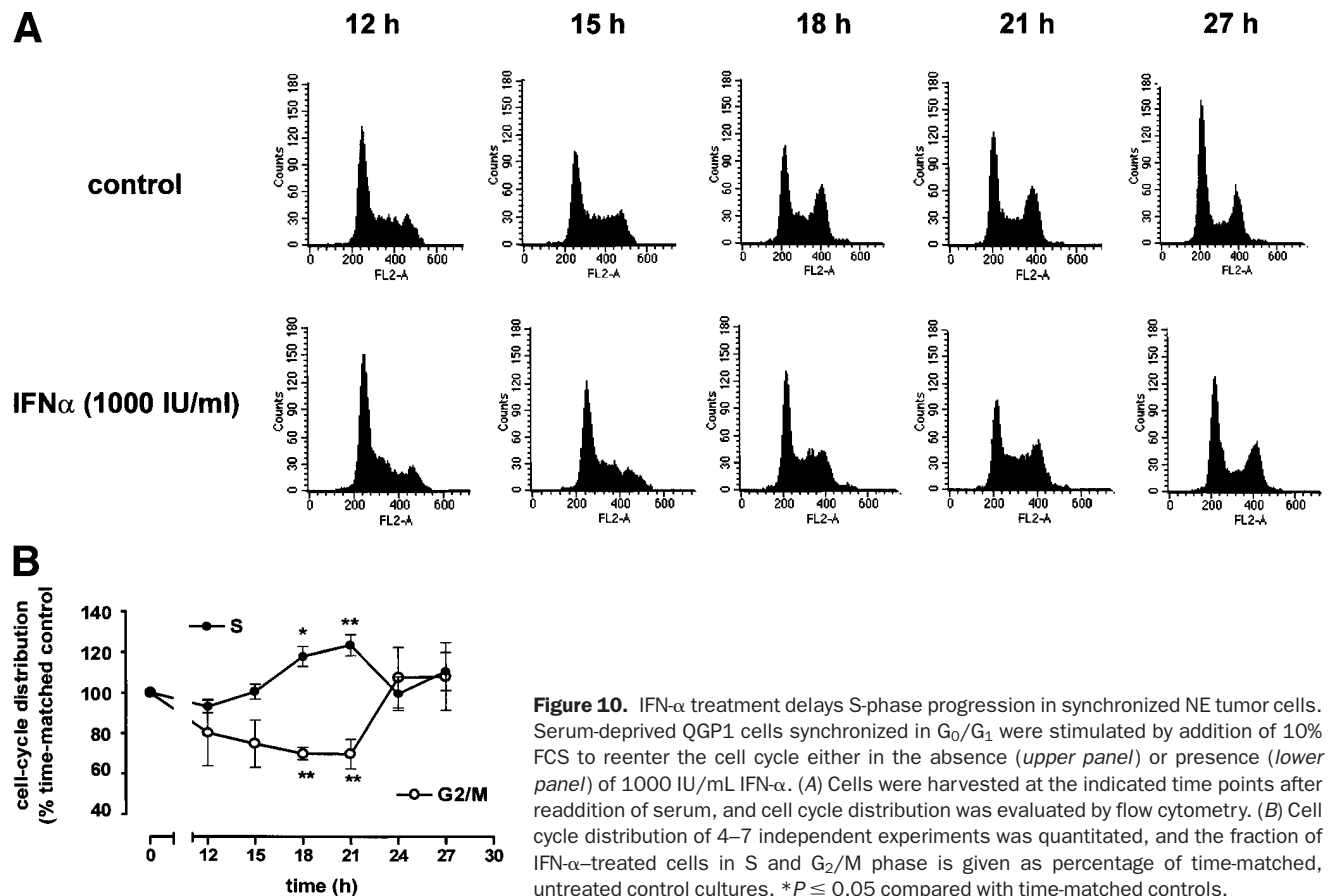


Figure 10. IFN- α treatment delays S-phase progression in synchronized NE tumor cells. Serum-deprived QGP1 cells synchronized in G₀/G₁ were stimulated by addition of 10% FCS to reenter the cell cycle either in the absence (*upper panel*) or presence (*lower panel*) of 1000 IU/mL IFN- α . (A) Cells were harvested at the indicated time points after readdition of serum, and cell cycle distribution was evaluated by flow cytometry. (B) Cell cycle distribution of 4–7 independent experiments was quantitated, and the fraction of IFN- α -treated cells in S and G₂/M phase is given as percentage of time-matched, untreated control cultures. * $P \leq 0.05$ compared with time-matched controls.

been determined. In contrast, the functional importance of Stat1 activation for the growth-modulatory actions of type I IFNs has been unequivocally established by studies in Stat1-deficient cells³³ and targeted gene disruption in transgenic mice.³⁴ Therefore, in the current study we confirmed Stat1 activation after IFN- α stimulation for both NE cell lines used. Previously, the duration of Stat1 activation has been identified as a critical determinant for the growth-inhibitory action of IFN- α in G₁-arrested Daudi cells.³⁵ Although minor differences in the time course of Stat1 activation as well as in the extent of Jak1 phosphorylation were noted between the 2 cell lines investigated, these did not translate into appreciable differences in the growth-regulatory effects of the cytokine.

IFN- α elicited direct inhibitory effects on anchorage-dependent and anchorage-independent growth in both NE cell lines. Our results thus confirm and extend a recent report of a direct antiproliferative effect of IFN- α in BON cells.³⁶ Specifically, IFN- α treatment prolonged cell doubling times, but even at maximal concentrations, the cytokine was unable to produce a cell cycle arrest or induce apoptosis, as suggested in hematopoietic and hepatoma cell lines, respectively.^{37,38}

A detailed analysis of IFN- α -induced changes in cell cycle distribution in both asynchronous and synchronized cell populations indicated that IFN- α specifically affected the S-phase progression. We failed to detect a functional impairment of G₁-to-S progression in either cell line. Thus, NE GEP tumor cells substantially differ from Daudi cells, the *in vitro* model most extensively used to investigate cell cycle effects of IFN- α , in which the growth inhibition results at least in part from functional activation of Rb. Several lines of evidence indicate that Rb-dependent mechanisms such as regulation of the cellular Rb complement¹⁷ or phosphorylation status^{19,39} are not involved in the antiproliferative effects of IFN- α in our NE tumor model. First, no qualitative difference in the biological response was noted between Rb-positive BON cells and QGP1 cells, which lack detectable Rb expression. Second, cell cycle analysis did not indicate any alteration of the G₁-to-S phase transition. Third, IFN- α treatment did not modulate Rb phosphorylation in growth-inhibited BON cells. Consistent with the lack of relevant G₁ checkpoint regulation by IFN- α in NE tumor cells, the cdk inhibitors p21^{cip1} and p27^{kip1}, which act as upstream regulators of IFN-induced, Rb-dependent growth inhibition in mouse

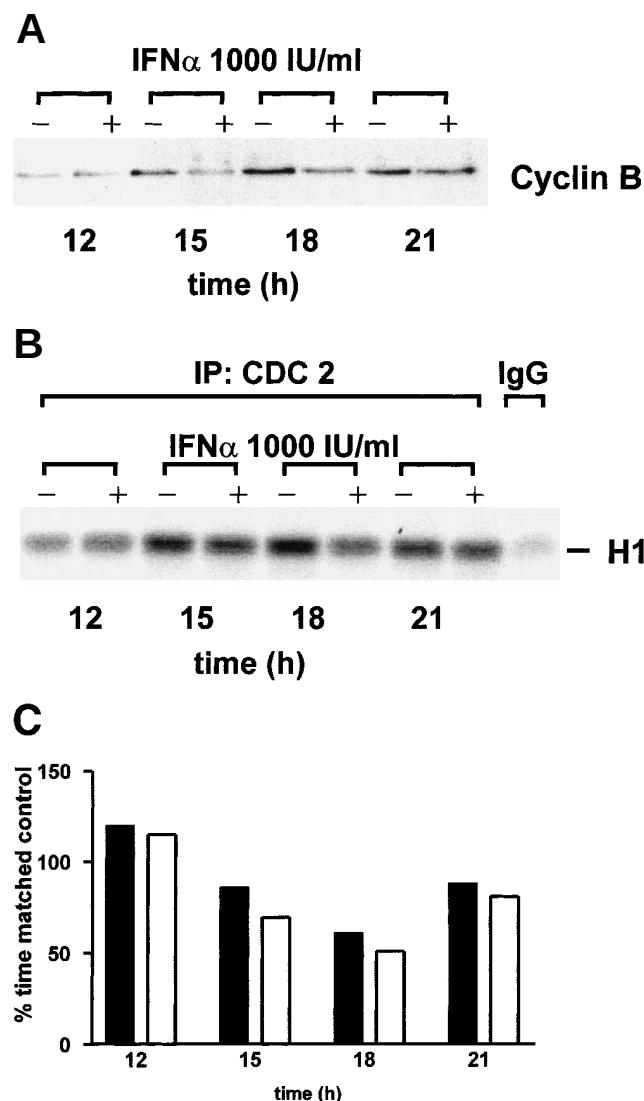


Figure 11. IFN- α -treated QGP1 cells fail to induce cyclin B, resulting in reduced CDC2 activity. (A) Whole-cell lysates of synchronized QGP1 cells were investigated for cyclin B expression by immunoblotting under control conditions or in the presence of 1000 IU/mL IFN- α . (B) Lysates from the same experiment were also used to immunoprecipitate CDC2 and subsequently assess CDC2-associated histone kinase activity. (C) Cyclin B expression ($\square$) and CDC2 activity ($\blacksquare$) were quantitated and the signal intensity was expressed as a percentage of the time matched, untreated controls. Results are representative of 3 independent experiments with similar results.

macrophages,⁴⁰ small cell lung cancer cells,⁴¹ Daudi cells,²⁰ and prostate cancer cells,⁴² were not induced in the current study.

In Daudi cells, induction of double-stranded RNA-dependent protein kinase (PKR) represents a functional component of the antiproliferative signal transduction pathway.²² This kinase was found to be up-regulated in IFN- α -treated BON cells as well as in biopsies of NE tumors from patients undergoing IFN- α treatment and has been implicated in its antiproliferative action.³⁶

However, because PKR is expressed in a cell cycle-specific manner with peak levels prevailing in the S phase of the cell cycle,⁴³ the reported PKR induction might rather reflect than mediate S phase-specific cell cycle effects such as those observed in the current study. Also, experiments in Daudi cells have identified c-myc as the critical growth-relevant downstream target of PKR, which in turn confers the G₁-phase specificity to the antiproliferative action of IFN- α .²³ The unhindered G₁-S progression observed in IFN- α -treated NE tumor cell lines does not support similar mechanisms in our model. Moreover, c-myc overexpression has been reported for both primary NE tumors²⁵ and BON cells⁴⁴ and may be partly responsible for the absence of G₁-specific cell cycle inhibition in our study.

Although most studies investigating type I IFN-induced cell cycle regulation observed effects on the G₁-to-S phase transition, S-phase cell cycle accumulation has been associated with growth inhibition in certain cell models both in vitro⁴⁵⁻⁴⁹ and in IFN- α -treated human colon cancer xenografts in vivo.⁵⁰ It has been suggested that this S-phase accumulation constitutes a response specific to transformed cells, where it arises from and reflects the defect in G₁ checkpoint control associated with the cellular transformation process.⁵¹ This concept is consistent with our present observation in NE tumor cells, where the defective G₁ checkpoint results alternatively from either loss of Rb (QGP1) or loss of p16^{INK4} (BON). However, the mechanisms that operate in the transformed cell models to cause S-phase accumulation have remained elusive. Our data on cell cycle distribution in synchronized QGP1 cells clearly show that the entry of IFN- α -treated cultures into the S phase of the cell cycle is not impaired. In subsequent steps of the cell cycle, however, the IFN- α -treated cells are deficient in their ability either to complete DNA replication or to transit into the G₂/M phase.

We now report a novel observation, i.e., a specific and selective delay of cyclin B induction in IFN- α -treated cells. As cells progress from S into G₂/M phases of the cell cycle, cyclin B is synthesized to function as a regulatory subunit in CDC2 kinase complexes.⁵² Consistent with this established function of cyclin B, we were able to demonstrate that the CDC2 kinase activity of human NE tumor cells is impaired in IFN- α -treated cultures with reduced cyclin B levels. Thus, our data argue in favor of an IFN-dependent effector pathway that specifically affects the S-G₂/M transition and propose cyclin B to be a critical target.

Cellular cyclin levels are typically tightly regulated both at the level of synthesis and degradation. The peak

of cellular cyclin B levels at the S–G₂/M transition is largely dependent on cell cycle inherent regulatory mechanisms. Specifically, cyclin B induction was shown to depend on CDK2 activity in experiments using cyclin B promotor constructs in NIH 3T3 cells,⁵³ thereby linking G₁ and G₂/M cell cycle transits. Thus, the lack of cyclin B induction observed in the current study may be caused by regulatory events earlier in the cell cycle. However, our analysis of CDK2 activity in both randomly cycling and synchronized NE tumor cells did not reveal a significant reduction. Moreover, the cellular content of CDC2, which represents another cell cycle-regulated gene product that depends on CDK2/E2F-mediated induction,⁵⁴ was not significantly affected by IFN- α in QGP cells (data not shown), but was down-regulated in IFN- α -treated Daudi cells as a result of reduced E2F activity.^{55,56} In contrast to the cell cycle changes observed in G₁ arrest models, our data are consistent with IFN- α specifically interfering with cyclin B induction.

A particularly intriguing aspect of the cell cycle-regulatory effects of IFN- α in NE tumor cells is their transient nature, causing an S-phase delay of approximately 6 hours compared with control cultures. In view of the substantial reduction of cellular proliferation it appears obvious that this delay in S-phase progression is maintained throughout various rounds of cell division. Therefore, it is difficult to contribute the transient kinetics of the cell cycle effects to regulatory events in the IFN-signaling pathway, such as receptor regulation or desensitization of intracellular effectors.³⁵ As an alternative possibility, IFN- α may affect but one of multiple, in part redundant, pathways that operate in the induction of cyclin B, which can be compensated for by alternative pathways—albeit with delay. Hence, a repeated retardation of cell cycle progression could occur, resulting in a pronounced reduction of cell numbers in the IFN- α -treated cultures. In this context, the cyclin B promoter offers alternative transcription start sites, which account for the constitutive expression throughout the cell cycle and the cell cycle-dependent rapid induction of cyclin B at the G₂/M-phase boundary, respectively.⁵⁷ Of note, sequences with similarity to octamer-binding motifs were described within proximity to the putative cell cycle-dependent start site,⁵⁸ and IFN- α is capable of down-regulating the cellular levels of octamer-binding factor in Daudi cells.⁵⁹

In summary, the current study provides a detailed analysis of the direct, cell cycle-regulatory effects of IFN- α in an in vitro model of NE tumor disease. We show a direct antiproliferative effect of IFN- α that results from a substantial delay in S–G₂/M phase transit. We

propose that selective inhibition of the cell cycle-dependent up-regulation of cyclin B results in reduced CDC2 activity and represents the functional basis of the observed cell cycle effects. Given the low proliferative fraction prevalent in endocrine GEP tumors, these mechanisms could translate into a clinically relevant stabilization of disease. Also, the ability of IFN- α to increase the S-phase population may offer the potential of chemosensitization when applied in combination with conventional cytotoxic or other biotherapeutic drugs.

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