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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 G_1 synchronized QGP1 cells. IFN-α–treated cells entered S phase in parallel to control cultures, but their progress into G_2/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 G_2/M. These cell cycle changes are associated with inhibition of cyclin B expression, resulting in reduced CDC2 activity.**

Neuroendocrine (NE) gastroenteropancreatic (GEP) tumors constitute a biologically heterogeneous group of ... 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.

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 induced growth regulation used Daudi Burkitt lymphoma cells and identified multiple signaling pathways that apparently function in parallel to block G_1–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 G_1 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 Rb[24] 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-^{32}P]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-α2b (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

1640 and DMEM, respectively, supplemented with 10% (vol/vol) FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and kept in 95% air and 5% CO_2 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 G_1-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 μL 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 μg/mL ribonuclease A, 0.1 % Triton X-100, 1 μmol/L EDTA, and 1.5 μg/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 (Na_3VO_4), 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 μmol/L phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL aprotinin, and 2 μmol/L leupeptin). Extracts were boiled in (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 (Biometra, 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 hypotone lysis (1 mmol/L $NaHCO_3$, 5 mmol/L $MgCl_2$, 0.1 mmol/L PMSF, and 20 μg/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 500*g*. Nuclei were further purified by saccharose gradient centrifugation (45% [wt/vol] saccharose, 30 minutes, 1660*g*) 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 μg/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 μg/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 μg/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 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 μg/mL aprotinin, 5 μg/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, 13,000*g*), lysates (500 μg/sample) were precleared for 1 hour by incubation with protein A–Sepharose beads. Immuncomplexes 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. Immuncomplexes 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 μmol/L ATP, and 5 μCi [γ-^{32}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 Bhattacharya[27] 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 RNAzol R reagent (WAK Chemie, Bad Soden, Germany) according to the manufacturer's 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, amplificates 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 control[29] (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 phophorylation by immunoblotting. After IFN-α stimulation, Jak1 immunoprecipitates from QGP 1 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

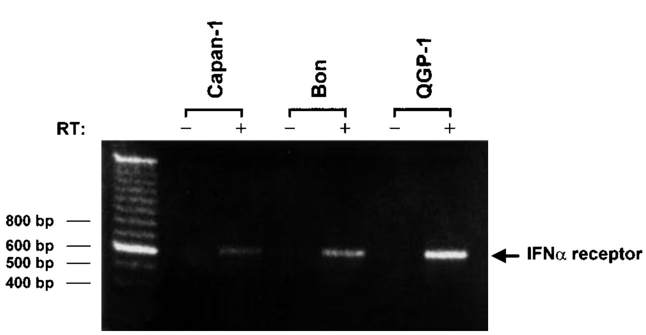

Figure 1. NE tumor cells express IFN-α receptor mRNA. Total RNA was extracted from QGP1, BON, and Capan1 cell lines, reverse-transcribed, and amplified by PCR using primers directed against the type I IFN receptor α chain. For size determination of the obtained PCR fragment, a 100-bp DNA standard was used (*lane 1*). Alternating

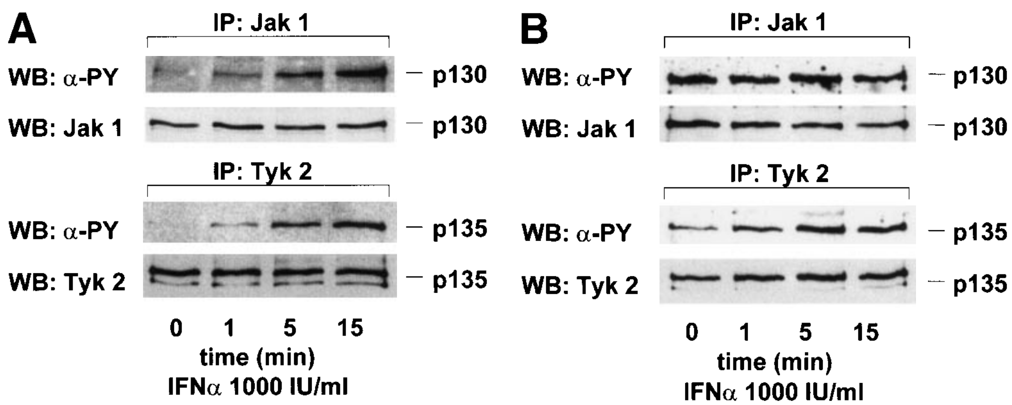

Figure 2. IFN-α treatment initiates activation of receptor-associated kinases in human NE tumor cells. Immunoblots of Jak1 and Tyk2 immunoprecipitates analyzing IFN-α–induced changes in the phosphotyrosine content of IFN-α receptor–associated kinases in (*A*) QGP1 and (*B*) BON cells. Cells were incubated 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 2*B*), 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 3*A*) and BON cells (Figure 3*B*), 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.

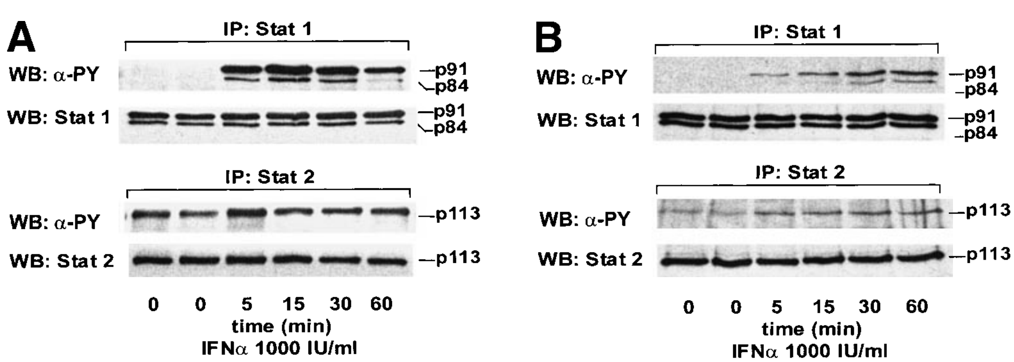

Figure 3. IFN-α time-dependently induces tyrosine phosphorylation of Stat1 and Stat2 in NE tumor cells. Both tyrosine phosphorylated (*upper row*) and total (*lower row*). Stat complement of Stat1 (*upper panel*) and Stat2 immunoprecipitates (*lower panel*) from IFN-α–treated cells were

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