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# IFN-$\alpha$5 Mediates Stronger Tyk2-Stat-Dependent Activation and Higher Expression of 2′,5′-Oligoadenylate Synthetase Than IFN-$\alpha$2 in Liver Cells

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## ABSTRACT

**Interferon-$\alpha$5 (IFN-$\alpha$5) is the main IFN-$\alpha$ subtype expressed in the liver. Hepatitis C virus (HCV) infection is associated with low IFN-$\alpha$5 mRNA levels, possibly reflecting an escape mechanism of the virus. In this work, we sought to compare IFN-$\alpha$2 and IFN-$\alpha$5 with respect to activation of early cell signaling cascades and induction of antiviral genes in the human hepatoma HepG2 and Huh7 cell lines. We found that the $Tyr^{701}$ phosphorylation kinetics of Stat1 mediated by IFN stimulation was higher when cells were incubated with IFN-$\alpha$5 than when using IFN-$\alpha$2. Similarly, $Tyr^{1054/1055}$ phosphorylation kinetics of Tyk2 were more intense after exposure to IFN-$\alpha$5 than when using IFN-$\alpha$2. Concomitantly, $Tyr^{705}$ phosphorylation of Stat3 was higher after stimulation with IFN-$\alpha$5 than with IFN-$\alpha$2. In parallel to these findings, the mRNA levels of the antiviral IFN-inducible gene *2′,5′-oligoadenylate synthetase* were higher in cell samples treated with IFN-$\alpha$5 than with IFN-$\alpha$2. These findings suggest that interaction of IFN-$\alpha$5 and IFN-$\alpha$2 subtypes with IFN type I receptor occurs differently, and this affects the intensity of expression of antiviral genes. In conclusion, our data show that in hepatocytic cells, IFN-$\alpha$5 induces stronger signaling and higher expression of antiviral genes than IFN-$\alpha$2. These data warrant clinical trials to evaluate the efficacy of IFN-$\alpha$5 in chronic viral hepatitis.**

## INTRODUCTION

Interferons (IFNs) are a group of cytokines with pleiotropic effects, including inhibition of cellular proliferation, induction of differentiation, modulation of the immune response, and activation of an antiviral status in the cell.[1,2] Human type I IFNs include a multigene family of different IFN-$\alpha$ subtypes and a single IFN-$\beta$. All type I IFNs are structurally related and share the same IFN receptor, with at least two subunits, IFNAR-1 and the full-length IFNAR-2c form.[1] The diverse activities of type I IFNs are mediated by conserved signal transduction pathways.[3,4] It is well established that binding of IFN-$\alpha/\beta$ to its receptor triggers signals that are transmitted through signal transducers and activators of transcription (Stats) from the cell surface receptor to the nucleus. Stimulation with IFN-$\alpha/\beta$ leads to tyrosine phosphorylation of the Jak1 and Tyk2 receptor-associated kinases. These two Janus kinases are responsible for the rapid activation of Stat2. Jak1 phosphorylates $Tyr^{701}$ in Stat1 and $Tyr^{690}$ in Stat2, which form an oligomeric complex called IFN-stimulated gene factor 3 (ISGF3) also containing a third protein p48, a DNA-binding protein. Tyrosine phosphorylation of Stat1 and Stat2 in response to IFN-$\alpha/\beta$ occurs in all nontransformed cells. Full activity of Stat1 (isoform Stat1$\alpha$) requires phosphorylation on $Ser^{727}$ probably via protein kinase C (PKC)-$\delta$/p38 mitogen-activated protein kinase (MAPK) pathway.[5–7] ISGF3 then translocates to the nucleus and activates the transcription of genes containing IFN-stimulated response elements (ISREs).

IFN-$\alpha/\beta$ also promote the formation of Stat1 homodimers, which bind to the IFN-$\gamma$ activation sequence (GAS).[8] Activation of Tyk2 kinase is essential to phosphorylate $Tyr^{705}$ in Stat3, and soon this factor is phosphorylated in $Ser^{727}$ to be fully activated.[9–11] Tyrosine phosphorylation of Stat3, Stat4, Stat5, and Stat6 by type I IFNs takes place in a cell type-specific manner.[4] Translocation of these transcription factors to the nucleus culminates in the activation of IFN type I-sensitive genes.[4] Of

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these genes, some are associated with regulation of apoptosis (caspases, Fas, p53), some with cell cycle arrest (p21, IFN regulatory factor-1 [IRF-1]), and some with immunoregulatory activities (IRF-1, IRF-3, and MHC class I).[2,12] The most intrinsic effect of type I IFNs, however, is their antiviral activity, which depends on the expression of few gene products. Those identified include the serine-threonine kinase RNA-activated (PKR), 2′,5′-oligoadenylate synthetase (2′,5′-OAS), the guanosine triphosphatase MxA, and P56, whose activation depends on the presence of viral dsRNA or ssRNA.[2,12,13] PKR and P56 proteins inactivate the eukaryotic initiation factors eIF2 and eIF3, respectively, and consequently cellular and viral protein synthesis is inhibited. The function of 2′,5′-OAS is to activate RNase L, which degrades and cleaves cellular and viral RNA. The Mx family of proteins mediates the inhibitory activity against replication of some viruses.

Most of the comparative signaling studies have analyzed the differences between IFN-$\alpha$ and IFN-$\beta$, and some divergence between these two IFN subtypes at the levels of Stat,[14,15] insulin receptor substrate-1 (IRS-1),[16] and Crkl[15] activation has been established. More recently, several groups have reported also that IFN-$\alpha$ subtypes can differ in promoting diverse signaling cascades after binding to the IFN receptor.[17–21]

We have found that IFN-$\alpha$5 is the only IFN-$\alpha$ subtype expressed constitutively in the liver and that the level of IFN-$\alpha$5 mRNA is markedly decreased in the livers of patients with chronic hepatitis C virus (HCV) infection.[22] This reduction in IFN-$\alpha$5 expression in HCV-infected livers appears to be a viral strategy to escape from the endogenous IFN-$\alpha$ system.

The aim of this study was to compare IFN-$\alpha$5 and IFN-$\alpha$2 with respect to signaling and the ability to induce antiviral genes in hepatocytic cells. Here, we show that IFN-$\alpha$5 induces not only a more potent activation signal but also a higher expression of 2′,5′-OAS than IFN-$\alpha$2 in Huh7 and HepG2 cells. These findings support the notion that these two IFNs may differ in their *in vivo* biologic effects on liver cells.

## MATERIAL AND METHODS

### *Cell culture*

HepG2 and Huh7 human hepatoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Gaithersburg, MD) supplemented with penicillin (0.6 $\mu$g/ml), streptomycin (60 $\mu$g/ml), glutamine (2 mM), and 10% fetal bovine serum (FBS).

### *Stimulation of cells with IFN*

Recombinant IFN-$\alpha$2b and IFN-$\alpha$5 were kindly provided by Dr. Vytautas Naktinis (Sicor Biotech UAB, Vilnius, Lithuania). Antiviral activity for IFN-$\alpha$2b is $1.67 \times 10^8$ IU/mg and for IFN-$\alpha$5 is $7.13 \times 10^7$ IU/mg. The purity of both IFN subtypes is >99%. HepG2 and Huh7 cells were seeded at 200,000/well in 6-well plates in DMEM plus 10% FBS. For signal transduction analyses, cells were serum starved for 8 h prior to IFN exposure. IFNs were used at 50 U/ml in the presence of 2% FBS for the periods indicated in each experiment.

### *Antibodies*

Antiphospho-Stat1$^{tyr701}$, antiphospho-Stat3$^{tyr705}$, antiphospho-Tyk2$^{tyr1054/1055}$ antibodies, and antirabbit IgG horseradish peroxidase (HRP)-linked antibody were purchased from Cell Signaling Bio-lab (Beverly, MA). Anti-Stat3 antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Stat1 antibody was from Santa Cruz Biotech, Inc. (Santa Cruz, CA). Antiactin and anti-Tyk2 antibodies were from Sigma-Aldrich (Steinheim, Germany) and Transduction Labs (Lexington, KY), respectively.

### *Western blotting*

After trypsinization, cells were collected by centrifugation. Cell pellets were resuspended and lysed in sample buffer containing dithiothreitol (DTT). Samples (50 $\mu$g protein) were resolved in SDS-PAGE under reducing conditions. Proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA) and stained with Ponceau red solution (Sigma-Aldrich) to verify equal loading of proteins. Membranes were incubated in TBS-T (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 0.1% Tween-20) with 5% dry milk. Proteins were detected by incubation with the specific antibody in TBS-T. After extensive washing in TBS-T, HRP-conjugated antibody was added for 1 h. Membranes were subjected to extensive washings in TBS-T, and the specific protein bands were visualized using the enhanced chemiluminescence (ECL) detection system (Perkin-Elmer, Boston, MA), according to the manufacturer's instructions. For reprobing, blots were stripped from membranes following the instructions of the manufacturer. Membranes were autoradiographed, and bands were quantified by densitometric analysis performed by Molecular Analyst/PC software (Bio-Rad Laboratories).

### *Analysis of mRNA expression by quantitative real-time PCR*

Total RNA was extracted from HepG2 and Huh7 cells using Ultraspec Reagent (Biotex, Houston, TX). RNA (1 $\mu$g) was treated with DNase (GIBCO-BRL) prior to reverse transcription with MMLV reverse transcriptase (RT) (GIBCO-BRL) in the presence of RNaseOUT (GIBCO-BRL). 2′,5′-OAS expression was measured by quantitative real-time PCR using a LightCycler and the LC-DNA Master SYBR Green mix (Roche Diagnostic GmbH, Mannheim, Germany). Aliquots of 2 $\mu$l from a 1:10 dilution of the cDNA pool were used for each PCR containing upstream and downstream primers specific for each gene in a 10-$\mu$l final volume. d(TTAAGAGGCAACTCCGATGG) and d(AGCAGACTGCAAACTCACCA) were the primers used to amplify a fragment of human 2′5′-OAS cDNA. d(AGCCTCGCCTTTGCCGA) and d(CTGGTGCCTGGGGCG) were the primers used for amplification of the reported human $\beta$-actin gene.

To determine the specificity, the PCR products were analyzed by melting curves. As an internal control for each sample, PCR amplification of a fragment of actin cDNA was performed. The amount of each transcript was expressed by the formula: $2^{cpactin-cpgene}$, where cp is the point at which the fluorescence rises appreciably above the background fluorescence.

### *Antiviral activity of IFN-α subtypes*

The antiviral activity of IFN-α subtypes was determined measuring the ability of IFNs to protect Huh7 and HepG2 cells from the cytopathic effect (CPE) of encephalomyocarditis virus (EMCV). The assay was performed in a 96-well microtiter plate. First, $2 \times 10^4$ Huh7 or HepG2 cells per well were seeded in 150 μl medium containing serial dilutions of IFN-α2 or IFN-α5 and incubated for 24 h. EMCV ($10^5$ PFU) was added to each well, and 24 h later, the CPE was measured as follows. After removing the medium, the wells were rinsed twice with phosphate-buffered saline (PBS) and stained with methyl violet dye solution (0.5% in 1:4 v/v methanol-water). The optical density (OD) was read at 540 nm. Results are expressed as percentage of cells protected against the CPE of EMCV.

### *Statistical analysis*

Statistical analysis was performed using nonparametric (Kruskal-Wallis and Mann-Whitney *U*) tests. All *p* values were two tailed and considered significant at $<0.05$. Descriptive data for continuous variables are reported as medians and interquartile range. SPSS 9.0 for Windows was used for the statistical analysis.

## RESULTS

### *IFN-α5 induces Stat1 activation more intensively than IFN-α2*

Activation of the Jak1-Stat1-dependent signaling pathway is required to initiate IFN-stimulated effects.[16] Thus, we compared the ability of IFN-α5 and IFN-α2 to activate Stat1. HepG2 and Huh7 cells were starved in DMEM for 8 h prior to IFN exposure. After stimulation with IFN, cells were collected at different times, and cell lysates were analyzed by Western blot with antibodies recognizing specifically Stat 1 or the tyrosine-phosphorylated form. Both IFN-α subtypes rapidly induced Stat1-$Tyr^{701}$. However, IFN-α5 induced a stronger Stat1-$Tyr^{701}$ signal than did IFN-α2 at 15, 30, and 60 min after stimulation (Fig. 1A,B). The densitometric values of the Stat1-$Tyr^{701}$ band were higher for IFN-α5 than for IFN-α2 at all the time points (Fig. 1C,D).

### *IFN-α5 induces higher Stat3 tyrosine phosphorylation than IFN-α2*

Stat3 is associated with the IFNAR-1 subunit, and its phosphorylation in tyrosine residue 702 depends on Jak1/Tyk2 kinases.[3,9] We compared the ability of IFN-α5 and IFN-α2 to

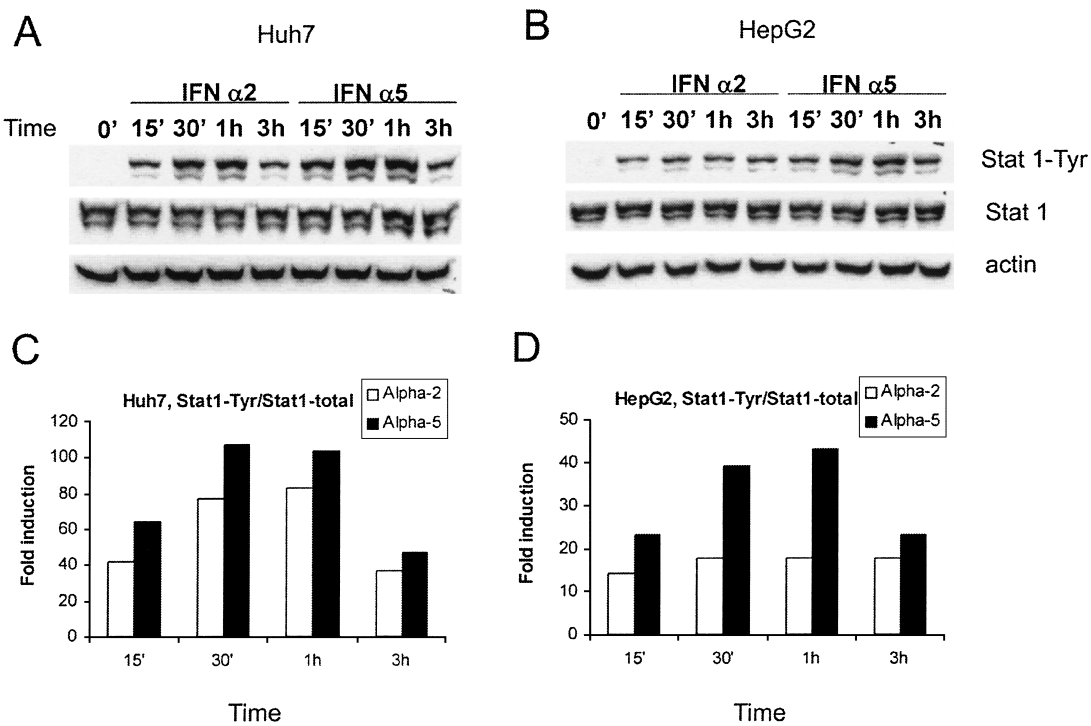


**FIG. 1.** Differential induction of Stat1-tyrosine phosphorylation by IFNα-2 and IFN-α5. (**A** and **B**) Huh7 and HepG2 cells were starved for 8 h and then incubated in medium plus 2% FBS in the absence (time 0) or presence of 50 U/ml IFN-α2 or IFN-α5 for the indicated times. Immunoblot analysis of total cell lysates for each treatment was assessed with the anti-Stat1-$Tyr^{701}$ antibody. The membrane was stripped, and the presence of total Stat1 protein was determined using an anti-Stat1 antibody. Corresponding samples were examined for actin concentration using antiactin antibody as the protein loading control. (**C** and **D**) Results are also expressed as the fold induction of the Stat1-Tyr/Stat1 ratio for each sample compared with the ratio obtained under untreated conditions. Results are representative of three independent experiments.

activate this transcription factor in HepG2 and Huh7 cells. Protein extracts from cells sampled before and at various times after the addition of IFN were analyzed using specific antibodies against Stat3 or Stat3-Tyr$^{705}$. IFN-$\alpha$5 induced a stronger activation signal of Stat3 15–30 min after stimulation in both cell lines (Fig. 2A,B). Densitometric values of the Stat3-Tyr$^{705}$ band were higher with IFN-$\alpha$5 than with IFN-$\alpha$2 15–30 min after stimulation in the two cell lines and also at 60 min in HepG2 (Fig. 2C,D).

### *IFN-$\alpha$5 induces higher Tyk2 phosphorylation than IFN-$\alpha$2*

The ability to activate the receptor-associated kinase Tyk2 by IFN-$\alpha$2 and IFN-$\alpha$5 was compared in these cell lines. Using an antibody that recognizes Tyk2 phosphorylation in the 1054/1055 Tyr position, we found that the induction of phospho-Tyk2 15–30 min after stimulation with IFN was stronger when Huh7 cells were incubated with IFN-$\alpha$5 than when using IFN-$\alpha$2 (Fig. 3A). In HepG2 cells, the induction of phospho-Tyk2 was higher 15–60 min after stimulation with IFN-$\alpha$5 with respect IFN-$\alpha$2 (Fig. 3B). Densitometric scanning showed that the phospho-Tyk2 bands were more intense with IFN-$\alpha$5 than with IFN-$\alpha$2 at 15–30 min in the two cell lines and also at 60 min in HepG2 (Fig. 3C,D).

### *Comparative analysis of expression of IFN-inducible genes by IFN-$\alpha$5 and IFN-$\alpha$2*

We next studied whether the differences found between IFN-$\alpha$5 and IFN-$\alpha$2 in their ability to activate Stat1 and Tyk2-Stat3 signaling pathways were paralleled by differences in their ability to stimulate the expression of antiviral IFN-sensitive genes. Thus, we measured by real-time quantitative PCR the mRNA levels of 2′,5′-OAS in HepG2 and Huh7 cells 9 h after stimulation with either IFN-$\alpha$2 or IFN-$\alpha$5. Both IFN-$\alpha$ subtypes were able to increase significantly the steady-state levels of mRNA of 2′,5′-OAS. Compared with IFN-$\alpha$2, IFN-$\alpha$5 induced significantly higher mRNA levels of 2′,5′-OAS in both cell lines ($p = 0.016$) (Fig. 4). In view of these results, the antiviral function of both IFN subtypes also was compared. At the dose used in this study, the two IFN subtypes did not differ in their ability to induce an antiviral status against EMCV in the two cell lines tested (Fig. 5).

## DISCUSSION

The interest in comparing IFN-$\alpha$2 and IFN-$\alpha$5 with respect to their effects on hepatocytes stems from our previous finding that the only IFN-$\alpha$ subtype detected in the liver is IFN-$\alpha$5.[22] In the current study, we present results indicating that IFN-$\alpha$5

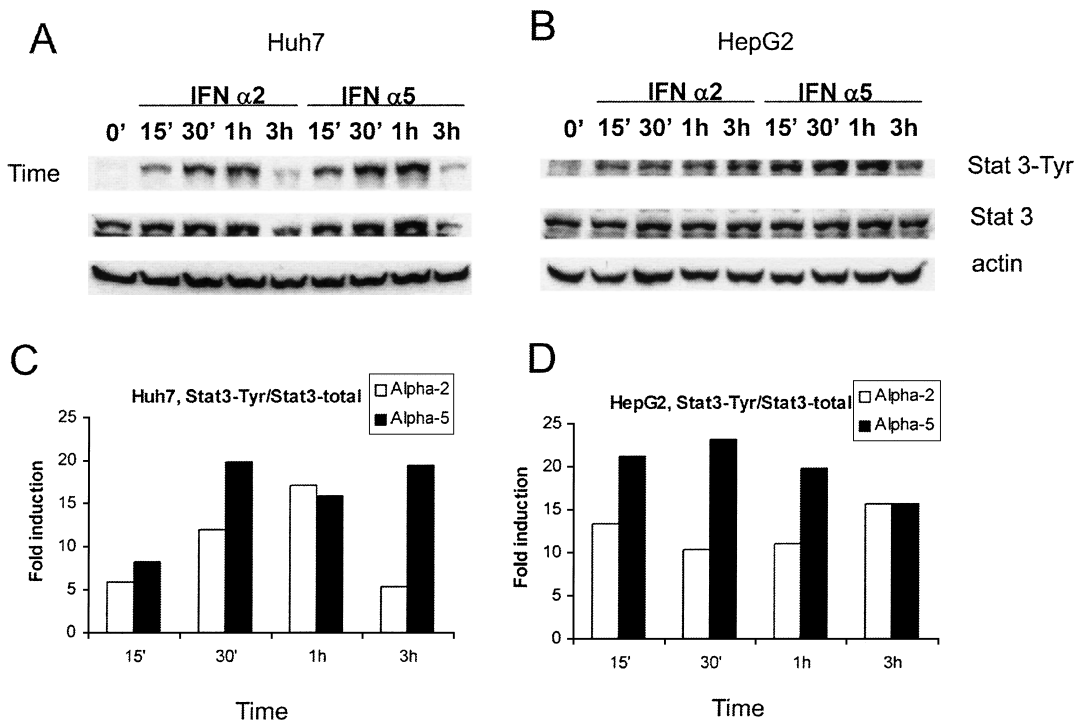


**FIG. 2.** Differential induction of Stat3-tyrosine phosphorylation by IFN-$\alpha$2 and IFN-$\alpha$5. (**A** and **B**) Huh7 and HepG2 cells were starved for 8 h and subsequently untreated (time 0) or treated with 50 U/ml IFN-$\alpha$2 or IFN-$\alpha$5 for the times indicated. Cell lysates were immunoblotted with phospho-Tyr-specific Stat3 antibody. The membrane was sequentially stripped and reprobed with antibody against Stat3 protein. Corresponding samples were also examined for actin concentrations using antiactin antibody as the protein loading control. (**C** and **D**) Results are also expressed as fold induction of the Stat3-Tyr/Stat3 ratio compared with the ratio obtained under untreated conditions. Results are representative of three independent experiments.

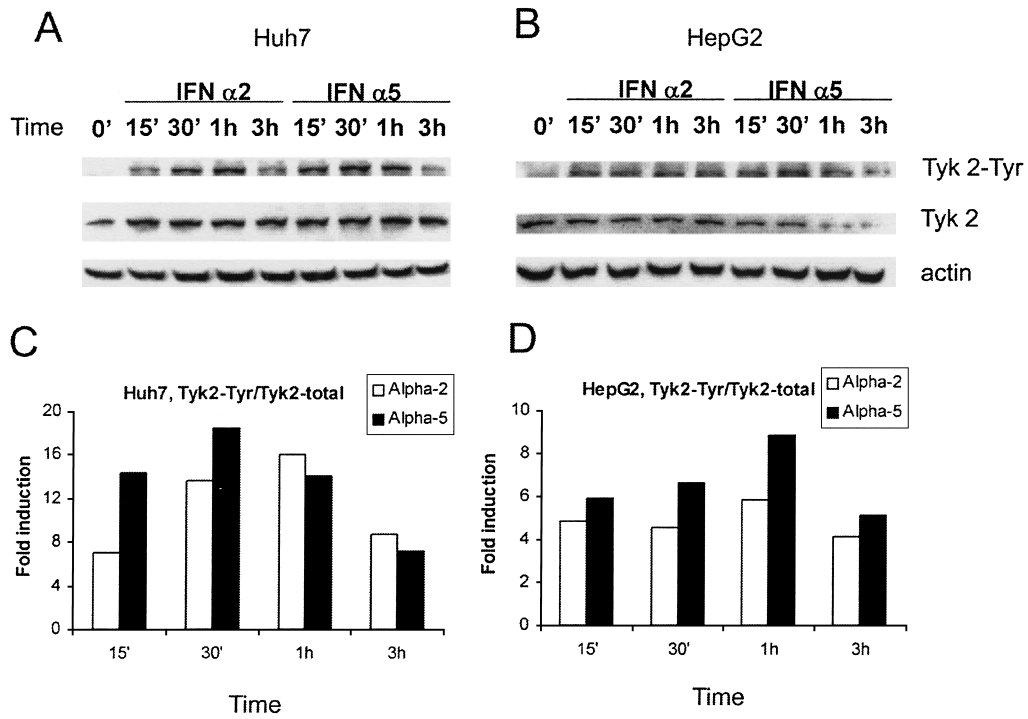


**FIG. 3.** Differential activation of Tyk2 by IFN-α2 and IFN-α5. (**A** and **B**) Huh7 and HepG2 cells were untreated (time 0) or stimulated with 50 U/ml IFN-α2 or IFN-α5 for the indicated times. An anti-Tyk2 phospho-Tyr-specific antibody was used to determine the Tyk2 phosphorylation state using whole cell lysates. The membrane was sequentially stripped and reprobed with antibody against Tyk2 protein. Corresponding samples were also examined for actin concentrations using antiactin antibody as the protein loading control. (**C** and **D**) The blots were subjected to densitometry, and the phospho-Tyk2/Tyk2 ratio changes relative to untreated cells are expressed as fold induction. Results are representative of three independent experiments.

induces a more potent activation of Stat1 and Tyk2-Stat3 pathways than does IFN-α2. These differences in signaling are accompanied by a more intense induction of 2′,5′-OAS by IFN-α5.

IFN-α subtypes show a close similarity at the structural level and exhibit a homology of 80%–100% in the amino acid sequence.[23,24] They all interact with the same receptor and induce similar biologic effects, including antiviral, antiproliferative, and immunomodulatory activities.[1,12] The reason for the presence of so many IFN-α subtypes with highly structural homology among them remains obscure. Recent data support the notion that the sites that bind to the receptor may differ among

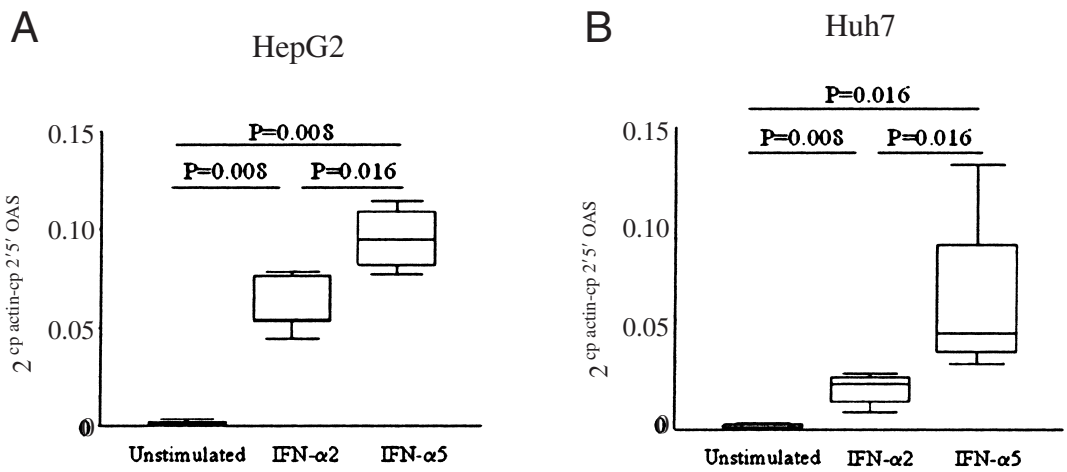


**FIG. 4.** Differential antiviral gene induction between IFN-α2 and IFN-α5. 2′,5′-OAS expression by real-time PCR in (**A**) HepG2 and (**B**) Huh7 cells unstimulated or stimulated for 9 h with 50 U/ml IFN-α2 or IFN-α5.

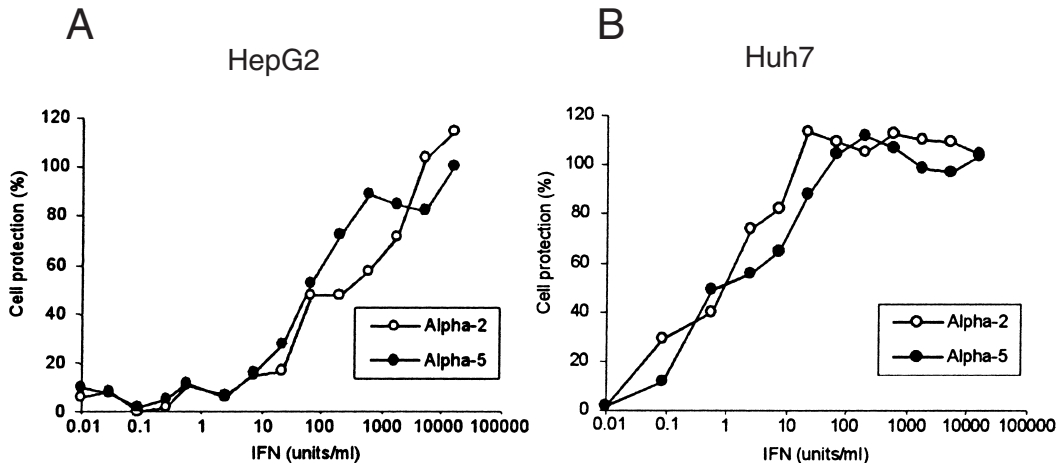


**FIG. 5.** Antiviral activity of IFN-$\alpha$2 and IFN-$\alpha$5. Percentage of cells protected by IFN-$\alpha$2 or IFN-$\alpha$5 against the cytopathic effect of EMCV in (**A**) HepG2 and (**B**) Huh7 cells.

subtypes and that some of them bind to the receptor with higher efficiency than others.[25,26]

Examination of the primary structure of IFN-$\alpha$2 and IFN-$\alpha$5 proteins reveals 85% homology between them, with 24 differences in amino acid residues. Notably, it has been shown that Arg at position 22 of the IFN-$\alpha$2 sequence is important with respect to antiviral activity.[27] In IFN-$\alpha$5, position 22 consists of Gly (a noncharged residue) instead of Arg (a positively charged residue), a change that could influence the electrostatic interaction with the receptor and intracellular signaling. Moreover, it has been proposed that residues 24–29 of the IFN-$\alpha$2 sequence are involved in binding to the receptor.[27] The replacement of Leu (a hydrophobic residue) at position 26 of IFN-$\alpha$2 by a Pro (a hydrophyllic residue) in IFN-$\alpha$5 may cause a change in the tridimensional structure of IFN that may modify the affinity for the receptor and the biologic activity. In fact, it seems that small variations in the primary sequence of the ligand may affect its interaction with the receptor, causing differences in the transmission of the signal.[27] In this regard, previous reports have shown that the intensity of biologic effects on specific cell types can be different for the diverse IFN-$\alpha$ subtypes,[27,28] inducing different antiviral and antitumor activities.[17–20,27,29,30] In a recent study, a group described several murine IFN-$\alpha$ subtypes that induce a diverse Stat activation that correlates with different *in vivo* responses against erythroleukemia.[21]

In agreement with our results, Yamamoto et al.[19] found that IFN-$\alpha$5 induced the highest levels of 2′,5′-OAS in human hepatocarcinoma KYN-3 cells. Additionally, it was observed that IFN-$\alpha$5 is as potent as IFN-$\alpha$8 (and more potent than IFN-$\alpha$2) with respect to protection against EMCV in hepatoma Huh7 cells.[19] In apparent contradiction to our results, Radaeva et al.[31] showed that multiple HuIFN-$\alpha$ species activate Stat1, Stat2, Stat3, and Stat5 to different extents in primary human hepatocytes, observing no important differences between IFN-A (IFN-$\alpha$2) and IFN-B (IFN-$\alpha$5).[31] We consider that these divergences could occur not only because of the use of different cell systems and IFN doses but, more importantly, because Radaeva et al. analyzed cell samples at only one time point after IFN stimulation. Our results clearly demonstrate that the activation of Tyk2 and Stat1 and Stat3 is stronger in HepG2 and Huh7 cells with stimulation with IFN-$\alpha$5 than with IFN-$\alpha$2 when analyzed at different times. Particularly, differences were more evident at 15–30 min after the addition of IFN, when IFN-$\alpha$5 demonstrated an ability to induce more intense phosphorylation in these molecules than IFN-$\alpha$2. Moreover, these observations fit with the higher expression of 2′,5′-*OAS* found in samples stimulated with IFN-$\alpha$5 with respect to those stimulated with IFN-$\alpha$2. Despite these differences, we did not observe more antiviral potency with one subtype than with the other in *in vitro* assays with the two cell lines infected with EMCV. It seems possible that the antiviral effects of different IFN-$\alpha$ subtypes vary according to the specific type of virus and also with the type of cell or tissue that is infected.

In conclusion, our data show that IFN-$\alpha$2 and IFN-$\alpha$5 exert different biologic responses on hepatic cells *in vitro*. This observation offer grounds for clinical trials aimed at determining the relative efficacy of IFN-$\alpha$5 in the treatment of chronic viral hepatitis.

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