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Interferons are cytokines that have been shown to affect a wide variety of cellular functions, including inhibition of cellular proliferation, antiviral activity, immune regulatory functions, and activation of multiple cellular genes (1). Antiviral and antiproliferative activities are clinically important properties of IFN-αs. Several types of IFN-α2 are currently approved for a variety of diseases including hepatitis b and c, hairy cell leukemia, and chronic myelogenous leukemia (2).

Recombinant techniques are useful methods for the production and modification of IFN-α proteins. The first IFN hybrid, IFN-α1/α2 was constructed using recombinant technology in 1981 (3). Subsequently, a number of hybrids have been constructed and have been informative for examining the activity of IFNs (4–11). These hybrid constructs have resulted in novel IFNs that either combine different biological properties from the parental proteins or have significantly different biological activity from both the parents (3). Therefore, IFN hybrids have provided a powerful tool for studying the structure-function relationship of these molecules. The engineered IFN-α proteins may have important new therapeutic applications and may provide greater insights into understanding of the clinical activities of existing IFN-αs.

To date, our laboratory has purified and characterized 22 IFN-α components produced by Sendai virus-induced human lymphoblastoid cells (12). One of these species, component o, was found to be noteworthy for its high antiproliferative activity and its poor ability to compete with the IFN-α2b binding site (13). Based on our partial amino acid sequence data, component o was indistinguishable from IFN-α2a. Therefore, rIFN-α2a was cloned and expressed. It was determined to behave functionally like component o in that it exhibits a high antiproliferative sp. act. and competes poorly for the IFN-α2b binding site (13, 14).

The goals of our studies are to determine the specific region(s) of IFN-α2a responsible for the enhanced antiproliferative activity and to further understand the signaling mechanism it uses to elicit this response. Three IFN2A and IFN2A21 chimeras (HY-1, HY-2, and HY-3) were constructed using PCR technology and cloned into a pQE-30 expression vector. The hybrid proteins were expressed in Escherichia coli. These chimeras were selected based on the 1) amino acid sequences of IFN-α2c and IFN-α2a, 2) the biological activities and binding properties of these IFN-αs, and 3) information derived from the three-dimensional structures of human IFN-α consensus and human IFN-β (15, 16). The three-dimensional model of a human IFN-α consensus sequence reveals that the two domains 29–35 and 123–140 are in close spatial proximity and may constitute a receptor recognition domain. In contrast, the residues 78–95 are distant from this region on the molecule and may influence species specificity and differential biological activities of the human IFN-αs and IFN-β (14).

The human IFN hybrid proteins were purified using nickel-nitrilotriacetic acid. Agarose and mAb affinity chromatography. The antiviral, antiproliferative, binding, signal transduction, and RNase protection properties of the IFN-α hybrids were analyzed and compared. Our data suggest that a portion of the N-terminal region of the molecule is important for interaction with receptor components, while the C-terminal region of IFN-α is critical for antiproliferative activity. The EMSA and RNase protection assays of the

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1 The sequences presented in this article have been submitted to GenBank under accession numbers HY-1 AF085803, HY-2 AF085804, and HY-3 AF085805.

2 Address correspondence and reprint requests to Dr. Renqiu Hu, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD 20892.

3 Abbreviations used in this paper: MDBK, Madin-Darby bovine kidney; GRR, γ response region; ISRE, IFN-stimulated response element.

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hybrid IFN-αs suggest that the antiproliferative response may not be directed by STAT1 and 2 activation alone in either a non-hematopoietic cell line, a hematopoietic cell line, or in a primary human cell type.

Materials and Methods

Expression and purification

IFNA2, IFNA21, and hybrids HY-2, HY-3 plasmid DNAs were individually transformed into E. coli strain SGI1000[pREP4] (Qiagen), and HY-1 plasmid DNA was transformed into E. coli strain DH5α/rIQ (Life Technologies, Gaithersburg, MD). Bacteria were grown in Luria-Bertani broth containing 100 μg/ml ampicillin (HY-1) or 100 μg/ml ampicillin and 25 μg/ml kanamycin (HY-2, HY-3, IFNA2, and IFNA21) in a 37°C shaker incubator overnight. The cultures were diluted 1:50 in Luria-Bertani broth containing the appropriate antibiotic(s) and incubated at 37°C with shaking to an A600 of 0.8–0.9. Protein expression was induced by 2 mM isopropyl-1-thio-β-D-galactosidase. The bacteria were then incubated at 30°C for 4–5 h, after which cells were harvested and sonicated. The cell lysate was clarified by centrifugation at 10,000 × g for 30 min at 40°C. IFN purification was performed by nickel-nitriotrifluoracetic acid. Agrose resin metal-affinity chromatography (20, 21) and 4F2 mAb affinity chromatography (12). The mAb 4F2 recognizes amino acid residues 113–149 of IFN-α consensus (Dr. Bruce Altrock, unpublished observations).

Cell and cell culture

Human Daudi cells were obtained from Dr. P. Grimley (Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD). Cells were grown in suspension using RPMI 1640 with 10% FCS, 2 mM glutamine, and 0.2% gentamicin. WISH cells (American Type Culture Collection (ATCC), Manassas, VA) were grown as monolayer cultures using Eagle’s minimal essential medium supplemented with 10% FCS and gentamicin (50 μg/ml). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. All cultures were determined to be free of mycoplasma. We obtained primary human lymphocytes from normal donors by centrifugal elutriation after Ficoll-Hypaque sedimentation (lymphocyte separation medium package insert, Organon Teknika, Durham, NC). The resultant cells were reseeded in RPMI 1640 media supplemented with 10% FCS and fungizone (250 ng/ml amphotericin B, 100 U/ml penicillin, and 100 μg/ml streptomycin).

Construction of pQE30/A21

A pair of oligonucleotides, 5'-TTCGATCTGTCTGTCGCTCCAGA C3'-sense and 5'-GAGCTCGATCTCAGTCATCCCTTCCTCCA ATCTC3'-antisense, with BamHI and SpII restriction sites were synthesized based on the cDNA coding region for mature human IFN-α21 protein and were used as primers. Human IFNA21 cDNA was used as the template. PCR was used to amplify an entire coding region for mature human IFN-α21 protein. The PCR reactions were conducted by standard procedures (17), and the resulting products were cleaved with restriction endonucleases BamHI and SpII and cloned into the E. coli expression vector pQE30 (purchased from Qiagen, Chatsworth, CA). The final construct was confirmed by DNA sequencing (18).

Construction of IFN-α hybrids DNA

Hybrid IFN cDNAs were constructed by PCR technology (19). PCR primers for the construction of HY-1 [IFN-α 21a(1-75)/IFN-α 2c(76-165)], HY-2 [IFN-α 21a(1-95)/IFN-α 2c(96-165)], and HY-3 [IFN-α 2c(1-95)/IFN-α 21a(96-165)] include: primer 1, 5'-TCC GGA TGC TCT GAG ACC CCT-3'(for HY-1, 2, and 3); primer 2, 5'-AGC AGA AGA GTC CTT TGT GGA GAG G3'-3'(for HY-1); primer 3, 5'-GCA TGC GAA GAC TGA TCT GCT-3'(for HY-2 and 3); primer 4, 5'-GAC AGA GTC TGC GAA GAG G3'-3'(for HY-1); primer 5, 5'-CAC GCA GGC TTC GAC ATC CAG-3'(for HY-2 and 3); primer 6, 5'-CTG AAT GAT CTC GAC GCC TCC GTG-3'(for HY-2 and 3); primer 7, 5'-GAG CTC GCA TGC TCA TCA TTC CTT CTT CTC TAA TCT-3'(for HY-3). Primers 2, 3, 5, and 6 were used as inside primers for all three hybrids, and primers 1 and 4 (HY-1 and HY-2) or 1 and 7 (HY-3) were used as outside primers. The BamHI restriction site is on the 5' outside primer (primer 1), and the SpII restriction site is on the 3' outside primer (primers 4 and 7). The DNA sequences of IFNA2 and IFNA21 are identical in regions recognized by primer 1, hence, this primer was used for the construction of both IFNA2 and IFNA21 fragments. Plasmid DNAs Bluescript/A2 and pQE30/A21 were used as the templates for the first PCR reactions for all hybrids. Purified DNA fragments from the first PCRs were mixed as templates, and primers 1 and 4 (for HY-1 and 2) or primers 1 and 7 (for HY-3) were used for the secondary PCR reactions.

Expression and purification

IFNA2, IFNA21, and hybrids HY-2, HY-3 plasmid DNAs were individually transformed into E. coli strain SGI1000[pREP4] (Qiagen), and HY-1 plasmid DNA was transformed into E. coli strain DH5α/rIQ (Life Technologies, Gaithersburg, MD). Bacteria were grown in Luria-Bertani broth containing 100 μg/ml ampicillin (HY-1) or 100 μg/ml ampicillin and 25 μg/ml kanamycin (HY-2, HY-3, IFNA2, and IFNA21) in a 37°C shaker incubator overnight. The cultures were diluted 1:50 in Luria-Bertani broth containing the appropriate antibiotic(s) and incubated at 37°C with shaking to an A600 of 0.8–0.9. Protein expression was induced by 2 mM isopropyl-1-thio-β-D-galactosidase. The bacteria were then incubated at 30°C for 4–5 h, after which cells were harvested and sonicated. The cell lysate was clarified by centrifugation at 10,000 × g for 30 min at 40°C. IFN purification was performed by nickel-nitriotrifluoracetic acid. Agrose resin metal-affinity chromatography (20, 21) and 4F2 mAb affinity chromatography (12). The mAb 4F2 recognizes amino acid residues 113–149 of IFN-α consensus (Dr. Bruce Altrock, unpublished observations).

Protein determination

Purified recombinant protein concentrations were determined using the coomassie plus protein assay (Pierce, Rockford, IL). Purity of the rIFN-αs was assessed by SDS-PAGE and HPLC analysis (data not shown).

Antiproliferative assay

The antiproliferative activities of the IFN-α constructs were analyzed using human Daudi and WISH cells and primary human lymphocytes. The assays on Daudi cells were performed as previously described (13). The assays on WISH cells were performed by incubating the cells with various IFN-αs at the indicated concentrations for 72 h at 37°C. Fifty microliters of 2 mg/ml tritiated thymidine were added into each well and incubated for 4–6 h at 37°C. Then, 10% SDS, 0.01 N HCl (250 μl) was added to each well and incubated overnight at 37°C. The OD570 of each well was determined, and the percentage of growth inhibition was calculated by comparing the control cultures (no IFN) with the IFN-treated cultures. Primary human lymphocytes were treated with PHA (Promega, Madison, WI) at 1 μg/ml overnight. The resultant PHA blasts were then treated for 72 h with the various IFNs at the concentrations indicated (see Fig. 2). Percent inhibition was calculated from direct cell counts that were done by Coulter counter analysis (Coulter, Palo Alto, CA).

Antiviral assay

Antiviral activity was determined as previously described using MDBK cells (ATCC) and WISH cells (ATCC) (12). All IFN units are expressed with reference to the National Institutes of Health human lymphoblastoid IFN standard Ga 23-901-532.

The Edmonson strain of measles virus (low passage, human embryonic kidney 7,vero5) was plaque purified and used to infect 1 × 10⁶ primary human lymphocytes (in triplicate) following no treatment or pretreatment with 100 ng/ml of parental or hybrid IFN for 24 h before infection as previously described (22). Cells were infected with measles virus at 0.1–1.6 multiplicity of infection or mock infected with virus-free medium and harvested 72 h postinfection. Cell-associated virus and supernatants were titrated on VERO cell monolayers. Measles virus cytopathic effect was evaluated microscopically after 6 days and confirmed by staining with crystal violet. The results reported are a comparison of the mean virus in supernatants harvested from the untreated group with supernatant virus in each treated group.

EMSA

Cells were treated with the various IFNs at the concentrations and times indicated (see Fig. 4) and centrifuged at 1500 × g at 40°C for 10 min. After being washed once in ice-cold PBS, the cells were re centrifuged at the above conditions. The cell pellet was then resuspended in lysis buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM MgCl₂, 150 mM NaCl, 1% Triton X-100, 3 mM sodium orthovanadate, 10 mM B-glycerophosphate, 20 mM DTT, and 2 mM PMSF) followed by centrifugation for 5 min at 14,000 × g. EMSA were then performed. The clarified whole-cell lysates containing 10 μg of total protein were diluted 1:1 in a binding buffer containing 20% glycerol, 2 mM DTT, 150 mM KCl, 2 μg polyoxyethylene -bisoxtecytosine, 0.5 mM EDTA, and 0.5 mM MgCl₂ and incubated for 15 min on ice. One nanogram of 32P-end labeled probe was then added to the reaction and allowed to sit an additional 5 min on ice. For the nuclease experiments, nuclease STI (New England Biolabs), or another rabbit serum was then added at this point at a 1:2000 final dilution (in binding buffer) and was allowed to incubate for an additional 30 min on ice. The samples were then subjected to electrophoresis on a native 6%,
TABLE I. Summary of antiproliferative, antiviral, and binding activities of hybrid and parent IFNs

<table>
<thead>
<tr>
<th>IFN</th>
<th>Antiproliferative Activity (ng/ml)</th>
<th>Specific Antiviral Activity (IU/mg)</th>
<th>Binding Activity (competes with 125I-IFN-α2b) (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Daunti cells</td>
<td>Wish cells</td>
<td>MDBK cells</td>
</tr>
<tr>
<td></td>
<td>Ratio wish/Daunti</td>
<td>MBDK cells</td>
<td>Wish cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MBDK/Wish</td>
</tr>
<tr>
<td>IFN-α2c</td>
<td>0.005</td>
<td>80</td>
<td>1.6 × 10^4</td>
</tr>
<tr>
<td>IFN-α21a</td>
<td>0.0008</td>
<td>95</td>
<td>1.2 × 10^5</td>
</tr>
<tr>
<td>HY-1</td>
<td>0.5</td>
<td>110</td>
<td>2.2 × 10^2</td>
</tr>
<tr>
<td>HY-2</td>
<td>1.0</td>
<td>&gt;1000</td>
<td>&gt;1 × 10^3</td>
</tr>
<tr>
<td>HY-3</td>
<td>0.0001</td>
<td>8</td>
<td>8 × 10^4</td>
</tr>
</tbody>
</table>

a Concentration of IFN species that inhibits cell growth by 50%.

b Concentration of IFN species that inhibits binding of 125I-IFN-α2b by 50%.

FIGURE 1. The amino acid sequences of IFN-α2c, IFN-α21, and IFN-α hybrids HY-1, HY-2, and HY-3. The amino acid leader sequence for all of these sequences is MRGSHHHHHHGS.

0.25× Tris-borate-EDTA gel for 4 h. The probes used consisted of the y response region (GRR) found within the promoter of the FcγRII gene (5′-AGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAG-3′) and the IFN-stimulated response element (ISRE) of the IFN-stimulated gene 1 (5′-GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC-3′) and the IFN-stimulated response element (ISRE) of the IFN-stimulated gene 15 (5′-ATCTATGCTCCTCGGAAGGGAACCCGAAACTGAGCC-3′).

RNase protection assay

This assay was performed as described previously (23).

Binding assay

IFN binding assays were performed as previously described on human Daudi (13) and WISH (24) cells.

Results

Purification and characterization of the IFN-αs and hybrids

Human IFN-α2c, IFN-α21a, and three IFN hybrids, HY-1, HY-2, and HY-3, were expressed in E. coli using a pQE30 expression system. The sequences of the IFN-αs deduced from their cDNA sequences are shown in Fig. 1. Initial purification of the IFNs on a Ni-NTA-Agarose column yielded partially purified material, and the sequences are shown in Fig. 1. Initial purification of the IFNs on a Ni-NTA-Agarose column yielded partially purified material, and the antiviral sp. act. of the purified IFNs ranged from 10^8 to 10^9 IU/mg on WISH cells. The antiviral sp. act. of HY-1 and HY-2 were, at least, 7-fold lower than that of HY-3, IFN-α2c, or IFN-α21a on WISH cells.

The ability of the IFN-α hybrids and the parental IFN-αs to inhibit the growth of Daudi, WISH, and primary human lymphocytes was also examined. The inhibition curves are shown in Fig. 2. The concentrations of IFN-αs that inhibited Daudi and WISH cell growth by 50% are shown in the Table I. Hybrid HY-3 always exhibited a higher antiproliferative sp. act. compared with the other hybrids and IFN-α2c and IFN-α21a on all three cell populations. In comparison, hybrid HY-2 had a lower antiproliferative sp. act., than either of the other hybrids or either of the parental IFN-αs on the same cell populations. HY-2 displayed 10,000-fold lower antiproliferative sp. act. compared with HY-3 on Daudi cells and >1,000-fold less sp. act. on WISH and primary human cells compared with HY-3. The hybrid HY-1 had a 2- to 8-fold greater antiproliferative sp. act. than HY-2 on Daudi and WISH cells. The relative ability of HY-2 and HY-3 to induce an antiviral state in a primary cell was determined by examining fresh human lymphocytes primed with PHA. The relative ability of HY-2 and HY-3 to induce an antiviral state in a primary cell was determined by examining fresh human lymphocytes primed with PHA. The antiviral sp. act. that for two separate normal donors at concentrations of HY-2 in which no antiproliferative effects were seen and STAT1 and STAT2 activation was readily apparent, there was suppression of measles virus. These data indicated that the antiviral pathway was operational. However, there was no effect on measles virus.
virus replication when titers of treated and untreated cell-associated virus were compared.

To examine the cell-surface binding properties of the hybrid molecules, competitive binding analysis was performed with IFN-α2c, IFN-α21a, the hybrids, and IFN-α2b. Fig. 4 shows the ability of IFN-α2c, IFN-α21a, and the three hybrids (HY-1, HY-2, HY-3) to compete for IFN-α2b binding sites on Daudi and WISH cells, respectively. IFN-α2c and the hybrid HY-3 inhibited the binding of 125I-IFN-α2b to Daudi and WISH cells very effectively, while hybrids HY-1 and HY-2 like IFN-α21a, poorly inhibited 125I-IFN-α2b binding on both cell lines. These data suggest that the amino terminal region of the IFN-α molecule is important in binding to its receptor.

IFN signaling pathways and transcription analysis

Because HY-3 has the highest antiproliferative activity and HY-2 has the lowest on Daudi and WISH cells and primary lymphocytes (Fig. 2), these two constructs were examined to explore potential signaling differences that could explain the dissimilarity seen in their antiproliferative activity. One signaling pathway that is activated by IFN-α is the JAK/STAT signaling pathway. Both STAT1

(7). A, Daudi cells. B, WISH cells. Cells were incubated in the presence of indicated concentrations of IFN for 3 days at 37°C. Results are expressed as a percentage of growth relative to a control culture to which medium without IFN was added. Experiments were performed in triplicate (Daudi) and duplicate (WISH). C, Primary human lymphocytes. Antiproliferative effects of the parental IFN constructs IFN-α2c (□) and IFN-α21a (△) and the HY-2 (○) and HY-3 (+) chimeras. Incubation with IFNs is same as for Daudi and WISH cells. Here direct cell counts were performed by analysis on a Coulter counter. The experiment was performed in triplicate and the results of the mean are represented.
and 2 are normally activated after IFN receptor occupancy. Therefore, to understand the mechanism governing this activity, we compared the ability of HY-2 and HY-3 to induce STAT1 and 2 activation by EMSA. Two 32P-labeled double-stranded oligonucleotide probes were used. One contains a GRR element that specifically binds STAT1, while the second contains an ISRE that binds STAT2. The data are shown in Fig. 5.

A

FIGURE 4. Competitive binding of IFN-α hybrids, IFN-α2c and IFN-α21a on Daudi (A) and WISH (B) cells. The concentration of 125I-IFN-α2b used in these experiments was 0.25 nM. Hybrids HY-1(△), HY-2(□), HY-3(●) and parent IFNs IFN-α2c(○) and IFN-α21a(●) were used as competitors. The experiments were performed in duplicate.

B

FIGURE 5. HY-2 and HY-3 both activate STAT1- and STAT2-containing DNA binding complexes. EMSA of extracts from HY-2- and HY-3-treated Daudi, Wish, and primary human lymphocytes are shown. Nuclear extracts (10 µg/lane) were prepared from 2 × 10⁷ cells treated for 30 min with either the HY-2 or HY-3 IFN construct at concentrations chosen from the antiproliferative profiles of each of the cells. For the Daudi cells, this was 0.1 pg/ml for HY-3 and 10 pg/ml for HY-2; for the Wish cells, this was 1 ng/ml for HY-3 and 10 ng/ml for HY-2; and for the primary human lymphocytes, this was 1 ng/ml for HY-3 and 100 ng/ml for HY-2. The extracts were incubated with radiolabeled GRR oligonucleotide previously shown to specifically bind IFN-α-activated STAT1 (A) or the ISRE oligonucleotide shown to specifically bind the IFN-α activated STAT2-containing ISGF3 complex (B).

FIGURE 6. Supershift analysis of EMSA of HY-2 and HY-3 IFNs. EMSA of whole-cell lysates from HY-2- and HY-3-treated primary human lymphocytes are shown and experiments performed as in Fig. 4 except that rabbit anti-STAT1 (top), anti-STAT2 (bottom), and normal rabbit serum Abs were added to the reaction and allowed to incubate on ice for 30 min before the addition of either radiolabeled GRR oligonucleotide (top) or radiolabeled ISRE oligonucleotide (bottom).
FIGURE 7. RNase protection assays using IFN-α2c, IFN-α21a, and IFN-α hybrids. The parental and hybrid IFNs constructs activate an IFN-α-inducible gene to the same extent at concentrations where antiproliferative activities of HY-2 are not observed. An RNase protection assay was performed using a radiolabeled riboprobe for the ISG15 gene (top) as a marker for the potential of each of the IFNs to induce transcriptional activation of an IFN-regulated gene. Then, 15 μg of total cellular RNA was isolated from cells treated for 16 h with the various IFNs at concentrations of 50 pg/ml, 5 ng/ml, and 100 ng/ml for the Daudi cells, Wish cells, and the primary PHA-treated human lymphocytes, respectively. A probe for GAPDH was included as a housekeeping gene control (bottom).

RNase protection assays were used to assess the ability of the parental and hybrid IFNs to induce several IFN-responsive genes. No differences were seen among the parental and hybrid IFNs in their ability to induce the ISG15 gene in Daudi or WISH cells, and the induction was comparable for HY-2 and HY-3 in PBLs (Fig. 7). The concentrations of HY-2 and HY-3 used were identical with those used in the EMSA experiments and were at levels where no antiproliferative activity was seen for HY-2 but good antiproliferative activity was noted for the other IFNs. In addition, the ability of the IFNs to activate IFN-induced gene expression was also identical for the ISG54, IRF1, GBP, and IP10 genes (data not shown). These results indicate that at concentrations where no antiproliferative activity was seen for HY-2, this construct retained the ability to induce a variety of IFN-inducible genes.

Discussion
One prominent effect of the IFN-αs is their ability to inhibit cell growth, which has also been suggested to be of major importance in determining antitumor action. Grander et al. (25) indicated that IFNs can exert profound antilcellular effects in vitro, caused by either cell cycle arrest and/or induction of apoptosis. Our studies show human IFN-α2c, IFN-α21a, and the hybrids HY-1, HY-2, and HY-3 exhibit a broad range (1,000–10,000) of differences in their antiproliferative sp. act. on Daudi and WISH cells and human lymphocytes (see Table I and Fig. 2). HY-3 exhibited the highest antiproliferative sp. act. The concentration of HY-3, which inhibits cell growth by 50%, is 0.0001ng/ml on Daudi cells and 8.0 ng/ml on WISH cells. However, these IFN-αs exhibited comparable antiviral sp. act. on MDBK cells and sp. act. within 7-fold of each other on WISH cells. Similarly, in primary human lymphocytes, the antiviral activity of HY-2 and HY-3 are essentially the same for the concentrations examined (see Fig. 6). Thus, the biological effects of HY-2 and HY-3 provide evidence for a divergent mechanism by which IFN-αs induce their antiproliferative and antiviral effects. Therefore, the IFN-αs may turn on several signaling pathways to different degrees (5). Recently, it has been reported that the antiproliferative effects of IFN-αα are mediated by components of TCR signaling pathways and that activation of the JAK-STAT pathway alone is insufficient for the induction of the antiproliferative activity of IFN-α (22). Our observations not only support that data but expand it beyond a cell line and a hematopoietic-based cell system. In addition our EMSA and RNase protection results suggest that the antiproliferative response may not be directed by activation of STAT1 and STAT2 alone. The gel shift data show that STAT1 and 2 activation and IFN-dependent gene transcription is induced by HY-2 at concentrations where HY-2 shows no antiproliferative activity. The antiviral activity was comparable between the two hybrids at concentrations in which no antiproliferative activity was seen. HY-3 was seen to induce STAT1 and STAT2 activation to a greater extent than HY-2 when the cells were treated with equivalent concentrations (data not shown).

However, the kinetic profiles of STAT1 and STAT2 activation by HY-2 and HY-3 at the various concentrations used in the antiproliferative assay were identical so that the differences seen between the two hybrids cannot be explained by STAT activation off rates (data not shown). Conversely, HY-2 exhibits little or no antiproliferative effects at concentrations that induce STAT1 and STAT2 activation more robust than that seen with low concentrations of HY-3 (data not shown).

Comparing the sequences of HY-1 and HY-2, there are seven amino acid differences in the 75–95 region (Fig. 1). Comparisons of published structural and homology models of IFN-β and -α (16, 26, 27), show that the amino acid region 75–95 forms the C helix, which is one of the most exposed helices. The amino acids in this region may be easily accessible to react with their appropriate receptors (28) and thus may be important for biological activity. Di Marco et al. (29) conducted site-directed mutagenesis in the C helix of the IFN molecule. They have reported that the mutated amino acids (position 84, 86, 87, 90) of helix C of IFN-α faces toward one of the subunits of the receptor and are part of one of two binding sites of IFN-α. Mutations markedly decreased the biological activity on murine cells compared with the unmutated protein. Korn et al. (15) and Fish (30) have proposed that there may be sufficient complexity in the type 1 IFN receptor to account for the differential sensitivities between IFN-αs and IFN-β that may be associated with a residue difference in the region 78–95. The IFN sequence 78–95 has been implicated as influencing species specificity among the murine and human IFN-αs and affecting the differential specificity of action between human IFN-αs and IFN-β. It is intriguing to speculate that these residue positions may constitute specific recognition sites associated with an accessory component of the IFN-α receptor. Our studies suggest that region 75–95 is very important for the antiproliferative activity of human IFN-α, with maximal activity observed when the construct has the α2c sequence from 75–95 and the α21a sequence from 96–166.

Our competitive binding experiments show that the hybrids or IFN-α with the α2c domain at the N terminus (residues 1–95; like IFN-α2c and HY-3) compete well with 125I-IFN-α2b binding. In contrast, hybrids or IFN-α with the IFN-α21a domain at the N terminus (residues 1–95; IFN-α21a, HY-1, HY-2) show reduced ability to compete with 125I-IFN-α2b binding. These data suggest that the N terminus is involved in IFN receptor binding interaction. Several reports have proposed two binding sites for IFN-α, one which is located in the N-terminal region and would determine the binding to high-affinity receptors. The second site would be in the C-terminal region of the IFN molecule and would influence low-affinity binding to cells (3, 31, 32). Extensive evidence suggests that this receptor system is complex, possibly consisting of either multiple receptors or a multisubunit receptor (13, 33).

In conclusion, our data demonstrates that a portion of the N-terminal region of the molecule may be important for the receptor binding activities. Furthermore, a domain critical for antiproliferative activity appears to exist within the C-terminal region from amino acid residues 75–166. In our studies the IFN region [α2c(75-95)/α21a(96-166)] appears to be responsible for the higher antiproliferative sp. act. seen with HY-3. In addition, our
data define IFN domains that dissociate antiviral and antiprolif-
erative activities. The hybrids (HY-1, HY-2, and HY-3) had sim-
lar antiviral sp. act. on MDBK cells and primary human lympho-
cytes but different antiproliferative and binding properties on
Daudi, WISH, and primary human cells. Analysis of gel shift gene
induction and biological responses suggest that the antiprolifera-
tive response requires critical signals in addition to those that ac-
tivate STAT1 and STAT2.

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