Characterization of N-Terminal Interferon τ Mutants: P26L Affords Enhanced Activity and Lack of Toxicity

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Interferon (IFN)-τ is a type I IFN that is responsible for the maternal recognition of pregnancy in ruminants. This protein also has classic IFN-like properties, including antiviral, antiproliferative, and immunomodulatory functions. Using IFN-τ as a model, we examined the structural basis for the activity of type I IFNs, focusing on amino acids within helix A and the first section of the AB loop, which have been proposed as a site for receptor interaction. Six amino-acid substitutions were made that replaced a residue in ovine IFN-τ1mod with the corresponding residue in human IFN-αA. Receptor binding was enhanced by a P26L mutation and was reduced by a conservative lysine-to-histidine substitution at residue 34. Alterations in the antiviral and antiproliferative activities of the IFN-τ mutants were not always correlated, but both functions were maintained or enhanced relative to the wild-type IFN-τ by the proline-to-leucine mutation at residue 26. In contrast, this mutation did not affect the low *in vitro* cytotoxicity that is characteristic of ovine IFN-τ1mod. Thus, the IFN-τ P26L mutant may have potential as an improved IFN-based therapeutic. Exp Biol Med 229:194–202, 2004

Key words: interferon τ; interferon α; antiviral; cytotoxicity; antiproliferative

Type I interferons (IFNs) are a family of proteins that possess antiviral properties, decrease cell proliferation, and play a modulatory role in the immune system (1). Members of the type I IFN family include IFN-α, IFN-β, IFN-δ, IFN-ω, and IFN-τ. IFN-τ was first discovered in sheep as a major conceptus protein that is produced and secreted in large amounts for a short time before implantation. Its role in sheep and other ruminants is to prevent regression of the corpus luteum by inhibiting transcription of the estrogen resceptor (2) and blocking the pulsatile secretion of prostaglandin F2α (3). This antiluteolytic protein was determined to have an amino-acid sequence with 45%–55% sequence homology with a range of IFN-αs and 70% with bovine IFN-ω (3). The protein sequences of the IFN-α and IFN-β contain 166 amino acids, and those of the IFN-α and IFN-δ contain 172 amino acids, with the additional six residues located at the C terminus (4, 5).

The sequence homology of the IFN-τ to the other type I IFNs prompted our assessment of its function. As has been observed for IFN-αs, different IFN-τ subtypes exhibit different relative antiviral and antiproliferative activities (6). Several subtypes of ovine IFN-τ have also been shown to have some degree of species cross-reactivity, such as that seen with human IFN-αB/D (7). Furthermore, the ovine IFN-τ1mod subtype has been used at high concentrations in vitro without producing a decrease in Madin Darby bovine kidney (MDBK) or peripheral blood cell viability (8, 9). *In vivo,* ovine IFN-τ1mod has effectively reduced the incidence and severity of murine experimental allergic encephylomyelitis (EAE) without decreasing animal weight, white blood cell counts, or lymphocyte function—complications that were observed with identical antiviral doses of murine IFN-α (10).

Human IFN-α2b and murine and human IFN-β have been crystallized and shown to possess similar three-dimensional structures (11–13). Recently, the crystal structure of ovIFN-τ1mod was determined at 2.1 Å resolution (14), and it too has a similar overall conformation to that of IFN-α and IFN-β. This core structure consists of five alpha helices (A–E) separated by loop regions (AB, BC, CD, and DE). Despite the overall structural similarity between the type I IFNs, significant differences also exist. The greatest variations occur at the N terminus (aa 6–7), helix A and the AB1 loop (aa 23–32), the AB3 loop (aa 49–52), and helix B and the BC loop (aa 71–77). These regions are located on one

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face of the molecule and are thought to be directly involved in receptor binding. Differential receptor binding may play a significant role in the different biological properties of type I IFNs (9).

The results of previous structure-function studies on IFN-α and -β support the working hypothesis that these regions are important for IFN activity. Extensive mutagenesis studies have pointed to loop AB as one of the "hot spots" for receptor binding and biological function (15), and mutations of human IFN-β and IFN-α at positions 27 and 35, as well as at position 123, have been shown to reduce antiviral activity (16). Studies that have used peptides corresponding to various regions of IFN-τ have shown that residues 1–37 inhibit the antiviral activity of ovine IFN-τ on MDBK cells but do not compete with huIFN-α2 to inhibit its activity (17). Thus, it has been suggested that antiluteolytic properties and reduced toxicity might be a function of particular N-terminal amino-acid residues. However, previous mutagenesis studies on IFN-τ focused on the C terminus (18, 19). Deletion of the C-terminal 11 residues did significantly decrease antiviral and antiproliferative activity but had only a slight negative effect on receptor binding.

In light of these studies, the present article focuses on the consequences of mutations in the N-terminal region of ovine IFN-τ1mod. Six mutations were constructed, three at sites within helix A and three within the AB loop. The mutations convert the specific amino acids in ovine IFN-τ1mod to the corresponding residues found in human IFN-αA. We have identified specific residues within helix A and the AB loop that affect antiproliferative and/or antiviral activity. The two activities are affected differentially by particular mutations, and changes in antiproliferative activity were cell type specific. One mutant, P26L, displayed antiviral and antiproliferative potency equivalent to that of the human IFN-α but maintained the lack of *in vitro* cytotoxicity of ovine IFN-τ1mod. Finally, none of the N-terminal mutants examined had altered cytotoxicity profiles, which suggests that this region may not be that which confers the observed nontoxic properties to ovine IFN-τ1mod.

Materials and Methods

Cell Lines. MDBK cells were cultured in minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and antibiotics. All of the human tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD). MCF-7 cells were grown in Eagle's MEM with 1 m*M* sodium pyruvate, l-glutamine, antibiotics, and 10% FBS. HT-29 cells were grown in Eagle's MEM supplemented with 0.1 m*M* nonessential amino acids, 1 m*M* sodium pyruvate, 10 μg/ml bovine insulin, l-glutamine, antibiotics, and 10% FBS. Daudi cells were grown in RPMI 1640 that contained 20% FBS.

IFNs. The gene encoding ovIFN-τ1mod was cloned into the methyltropic yeast *Pichia pastoris* (Invitrogen, San Diego, CA) under the control of the alcohol oxidase promoter (20). On induction with methanol, ovIFN-τ1mod was produced as a secreted protein. It was purified by ammonium sulfate precipitation followed by anion exchange chromatography using diethylaminoethyl cellulose (Sigma, St. Louis, MO). The specific activity of the purified protein was 1×10^8 U/mg. Recombinant human IFN-αA with specific activities of 3×10^8 and 1×10^8 U/mg, respectively, was purchased from Intergen (Purchase, NY) and PBL (New Brunswick, NJ).

Construction of ovIFN-τ1mod Mutagenesis/Expression Vector. For the construction of the ovIFN-τ1mod mutagenesis/expression vector, the gene for ovIFN-τ1mod was amplified by polymerase chain reaction (PCR) using *Taq* polymerase (Stratagene, La Jolla, CA) and cloned into the *Escherichia coli* vector pCR2.1 (Stratagene) before ultimately being cloned into the *Kpn*I site of the *E. coli*–yeast shuttle vector pPICZα (Invitrogen).

Site-Directed Mutagenesis. Six mutations were introduced using the Quickchange Site Directed Mutagenesis kit (Stratagene), according to the manufacturer's instructions. In brief, primers that contained the desired base changes were synthesized by Bioserve (Laurel, MD) or Integrated DNA Technologies (Coralville, IA). They were added to 50–100 ng of pPICZα that contained the gene for ovIFN-τ1mod with *Pfu* turbo polymerase (Stratagene) in a 50-μl reaction and cycled to incorporate the desired base change(s). Each reaction was optimized for each set of primers. Five microliters of the PCR reaction was run on a 1% agarose gel to visualize the product. The remaining reaction was digested with *Dpn*I for 1 hr, purified, and used to transform XL-1 Blue Ultracompetent cells (Stratagene). Transformants were selected on low-salt Luria broth (LB) in the presence of zeocin (Invitrogen). Plasmid DNA from transformants was extracted with phenol-chloroform and ethanol precipitated. The incorporation of the correct mutations was verified by dideoxy sequencing.

Production of Mutant IFN-τ Proteins in *P. pastoris*. *E. coli* carrying the recombinant plasmid was cultured overnight in low salt LB with zeocin and the plasmid DNA extracted. Plasmid DNA was linearized by digestion with *Sac*I overnight, purified, and resuspended in 5–10 μl of water. This DNA was used to transform *P. pastoris* either by electroporation or chemically by using the *Pichia* EasyComp kit (Invitrogen). One hundred microliters of the yeast transformation mix was plated on YPD (1% yeast extract, 2% peptone, and 2% dextrose) plates that contained zeocin and incubated at 30°C for 3 days to allow selection of yeast containing the desired gene. Individual colonies were selected and grown in 25 ml of BMGY media (1% yeast extract, 2% peptone, 100 m*M* potassium phosphate [pH 6.0], 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol). For the production of mutant proteins, cultures were shaken vigorously at 30°C in the presence of light to OD_{600} 2–6. They were harvested by centrifugation at 2500 *g* for 5 mins. The pellet was resuspended in

BMMY media (1% yeast extract, 2% peptone, 100 m*M* potassium phosphate [pH 6.0], 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% methanol) and again shaken vigorously at 30°C for 1–2 days to induce the expression of the proteins. Proteins were secreted into the medium and purified by ammonium sulfate precipitation and anion exchange column chromatography. Purified proteins were analyzed by electrophoresis on a 12% polyacrylamide gel, and each mutant showed only a single band by silver staining. The concentration of IFN-τ and mutant IFN-τ proteins was measured using the BCA protein assay (Pierce, Rockford, IL). The protocol was optimized for low concentrations of protein using an incubation period of 60°C for 30 mins.

OvIFN-τ1mod Mutant Protein Immunoblots. Purified proteins were run on a 12% polyacrylamide gel and transferred to Hybond membrane (Amersham, Piscataway, NJ). Membranes were incubated with a 1:500 dilution of the monoclonal antibody HL-98 made against a C-terminal region peptide of ovIFN-τ1mod. Recognition of both native and denatured ovIFN-τ1mod by this antibody was not dependent on a conformational determinant (21). The secondary antibody was peroxidase-conjugated sheep anti-mouse antibody. The proteins were detected using enhanced chemiluminescence, according to the manufacturer's instructions (Amersham).

Structural Determination. Circular dichroism (CD) of the IFN-τ mutants was determined at room temperature with a JASCO 500C spectropolarimeter. Scans were done with a 0.1-mm path length cell at a sensitivity of 2 and a time constant of 8 secs. The wavelength range measured was from 250 nm to 184–188 nm at a scan rate of 20 nm/min. Scans were carried out on IFN-τ mutants in water at 0.16–0.5 mg/ml. The CD spectra were expressed in terms of ellipticity, θ, related to the means residue molecular weight for each IFN-τ mutant. The following formula was used to generate θ (16): mean residue ellipticity $[\theta] = 100 \times [\theta]_{observed}/c \times l$, where $[\theta]_{observed}$ is expressed in degrees, c equals the mean residue concentration in mol/l, and l is the path length of the cell in cm. The three-dimensional structure of ovIFN-τmod1, resolved at 2.1 Å (14), was used to model mutant IFN-τs with the molecular graphics program Swiss-Prot DB Viewer (European Bioinformatics Institute, Heidelberg, Germany).

Competitive Binding of OvIFN-τ to Receptors on MDBK Cells. OvIFN-τ1mod was labeled with the Bolton-Hunter reagent (mono [^{125}I] iodo derivative, 2000 Ci/mmol, Amersham; 1 Ci = 37 GBq), as described elsewhere (9). The specific activity of the labeled protein was ~20 μCi/μg. The labeled ovIFN-τ1mod retained complete antiviral activity on MDBK cells. For binding, 3 n*M* of ^{125}I- ovIFN-τ1mod was incubated with 7.5×10^5 MDBK cells in the absence or presence of 300 n*M* unlabeled ovIFN-τ1mod, IFN-αA, or IFN-τ mutants in 500 μl of MEM/10% FBS at 4°C for 12–14 hrs (19). The cells were layered over 10% (w/v) sucrose in phosphate-buffered saline (PBS; 2.5 ml), centrifuged at 12,000 *g* for 30 mins at 4°C, and the pellets were counted. Specific binding was defined as total binding minus nonspecific binding in the presence of a 100-fold molar excess of unlabeled IFN-αA.

STAT1 Phosphorylation. Cells were incubated with medium or 1.7 n*M* IFN-α, ovIFN-τ1mod, or IFN-τ 26P:L for 10 mins. They were lysed and immunoprecipitated with anti-STAT1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), as described elsewhere (9). Immunoblots were done with either anti-STAT1 or anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY).

Antiviral Assay. Antiviral activity was measured using a standard cytopathic effect inhibition assay using MDBK cells and vesicular stomatitis virus (VSV; Ref. 22). Antiviral activity was normalized on the basis of the reference IFN-α Gxa01-901-535.

Cell Proliferation Assay. Antiproliferative activity was measured on two adherent cell lines, MCF-7 (breast adenocarcinoma) and HT-29 (colon adenocarcinoma), and one suspension cell line, Daudi (Burkitt lymphoma), using conditions previously optimized for each cell line. For the adherent cell lines, 1000 cells/ml were plated in a 24-well polysterene plate, and 10,000 units of ovIFN-τ1mod or the equivalent molar concentration (17 n*M*) of IFN-αA or IFN-τ mutants was added. Cells were incubated at 37°C in 5% CO_2 for 9 days. Cells were detached with 0.25% trypsin and counted using a hemocytometer. Viability was determined by trypan blue staining. For Daudi cells, 1000 cells/ml were incubated with 33 U of IFN-τ or the equivalent concentration (0.06 n*M*) of IFN-α or IFN-τ mutants in 5-ml polypropylene tubes. Cells were incubated at 37°C in CO_2 for 3–4 days, centrifuged for 5 mins at 300 *g*, and the pellet resuspended in 1% trypan blue in PBS and counted.

Cytotoxicity Assay. A total of 80,000 U/ml of either ovIFN-τ1mod, IFN-αA, or IFN-τ mutants was added to 2×10^5 U937 cells in polypropylene tubes in triplicate and incubated for 72 hrs. Control cells were treated with medium alone. Cells were counted with a hemocytometer after the addition of trypan blue.

Statistical Analysis. Statistical variations between means were determined by ANOVA, followed by the multiple comparison test of least significant difference (23). $P \leq 0.05$ was considered to be significant.

Results

Design of IFN-τ Mutants. It has been suggested that the N terminus of IFN-τ interacts with the type I IFN receptor in a distinct manner and is responsible for some of IFN-τ's unique activity (9, 17). Thus, we decided to focus on this region of the protein for further study. Our strategy involved changing particular amino acids in the well-characterized ovIFN-τ1mod to those in human IFN-αA. This allowed us to assess the contribution of individual amino acids to IFN-τ activity, as well as to see whether any of the residues created a molecule with activity more like that of IFN-α. Six mutations were chosen from amino acids

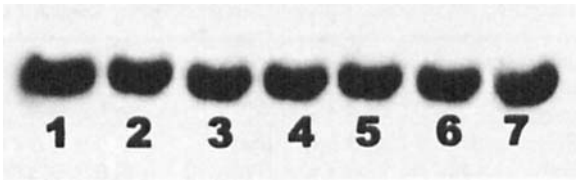

Figure 1. Immunoblot of IFN-τ mutant proteins. A 0.5-mg sample of IFN proteins was analyzed by immunoblot using a monoclonal antibody directed against the C-terminal region of IFN-τ. Lanes 1–7: IFN-τ, E13R, K16M, D19A, L24I, P26L, and K34H.

that were different between IFN-α and IFN-τ and exposed to solvent. The mutations were E13R, K16M, D19A, L24I, P26L, and K34H. The recombinant IFN-τ containing each mutation was expressed and purified. All mutant IFN-τ proteins were recognized by an anti–C-terminal monoclonal antibody in immunoblots (Fig. 1).

Binding to the type I IFN receptor. Each IFN-τ mutant protein was examined for its ability to compete with wild-type ^{125}I–ovIFN-τ1mod for binding to MDBK cells. The MDBK line was chosen because the binding affinities of both IFN-τ and IFN-α had been previously determined (9). Because human IFN-αA has a higher affinity for the type I IFN receptor, it was able to displace radiolabeled ovIFN-τ1mod better than the native protein itself, which produced only 50% displacement (Fig. 2). For this reason, specific binding was defined using IFN-αA as a reference. The majority of the substitutions introduced decreased the ability of the mutant proteins to compete off the labeled ovIFN-τ1mod (for L24I and K34H, this decrease was significant). However, the P:L substitution at residue 26 significantly increased the competition of labeled ovIFN-τ1mod binding, although not to the same extent as did the human IFN-αA. Phosphorylation of STAT1 subsequent to receptor interaction with IFN-τ, -α, or the P26L mutant was also examined (Fig. 3). IFN-α induced slightly higher levels of phosphorylated STAT1 than did ovIFN-τ1mod, and STAT1 phosphorylation was equivalent in ovIFN-τ1mod– and IFN-τ P26L–treated cells.

Antiviral Activity of IFN-τ Mutants. IFNs have historically been described by their antiviral activity, so this was a logical screen for the activity of the IFN-τ mutants. We tested the ability of the six mutants to protect MDBK cells against VSV and compared their activities with that of wild-type ovIFN-τ1mod and human IFN-αA. All of the mutants possessed antiviral activity to some extent, with the least active mutant, E13R, having 3.2×10^4 U of activity/mg protein (Fig. 4). This is significantly less antiviral activity than wild-type ovIFN-τ1mod (6.4×10^7 U/mg) or IFN-α (8×10^7U/mg). The reduced antiviral activity of E13R did not correlate with receptor binding, because the binding of E13R was equivalent to that of ovIFN-τ1mod. The activity of the IFN-τ P26L mutant was 9.5×10^7U/mg, which is as great as that of both IFN-τ and IFN-α. The antiviral activities of the four other mutants—K16M, D19A, L24I, and K34H—were significantly reduced relative to the parental IFN controls.

Antiproliferative Activity of IFN-τ Mutants. The ability of IFNs to decrease cell proliferation is a well-documented phenomenon. Therefore, we evaluated the antiproliferative activities of the IFN-τ mutants on three tumor cell lines, each of which is responsive to type I IFNs, although to varying degrees. In Daudi cells, human IFN-αA caused a 79% decrease in proliferation, compared with 42% for ovIFN-τ1mod (Table 1). All of the mutants tested reduced the cell numbers as well as or better than did ovIFN-τ1mod, with the P26L mutant exhibiting the greatest antiproliferative activity. This mutant caused an 80% decrease in cell number, compared with cells in medium alone, an activity profile that similar to that of IFN-α. Two adherent cell lines, HT-29 and MCF-7 (data not shown), were used to test the same property (Table 2). These cell lines are not as sensitive to type I IFNs, which correlates to an elevated EC_{50} and longer incubation time. All of the

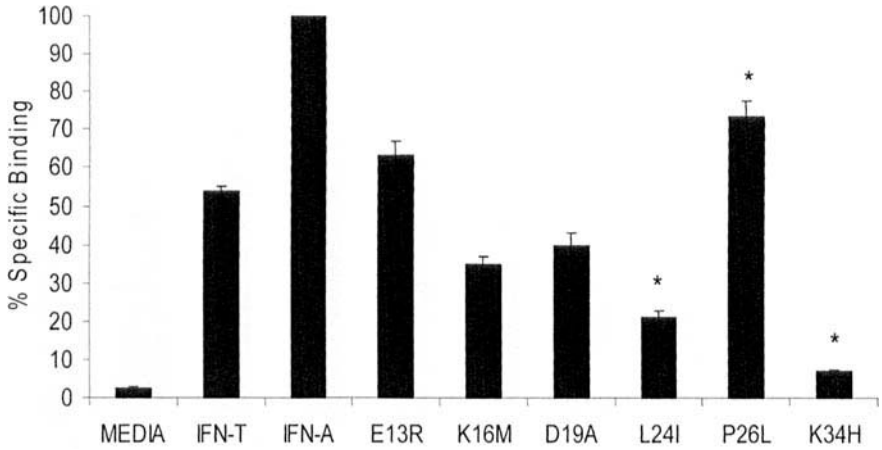

Figure 2. Binding of IFN-τ mutants to MDBK cells. ^{125}I-ovIFN-τ1mod (3 n*M*) was incubated with 7.5×10^5 MDBK cells in the absence or presence of 300 n*M* unlabeled ovIFN-τ1mod, IFN-αA, or IFN-τ mutants at 4°C for 12–14 h. Specific binding was defined as total binding minus nonspecific binding in the presence of a 100-fold molar excess of unlabeled IFN-αA. Results are expressed as mean % specific binding ± SE from two replicate experiments. *Significant difference determined by analysis of variance after arcsine transformation where $P < 0.05$.

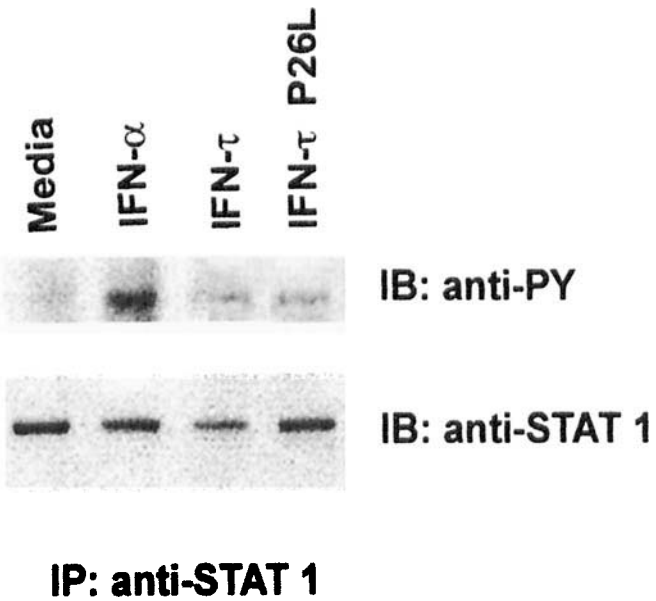

Figure 3. STAT1 phosphorylation by IFN-τ P26L. Cells were incubated with medium or 1.7 n*M* IFN-α, ovIFN-τ1mod, or IFN-τ P26L for 10 mins, lysed and immunoprecipitated with anti-STAT1, and immunoblotted with the indicated antibody.

mutants significantly decreased cell number compared with control, except the L:I mutation at position 24, despite the fact that this mutation is conservative. The P26L mutation significantly increased antiproliferative activity relative to wild-type IFN-τ, which suggests that this position contributes to overall function. All of the mutants inhibited proliferation of MCF-7 cells when used at 17 n*M;* however, the activity of the mutants did not significantly differ from that of ovIFN-τ1mod. As has been seen previously, the IFN-τ P26L mutant had the greatest antiproliferative activity, which was not significantly different from either of the parental IFNs.

Table 1. Antiproliferative Activity of IFN-τ Mutants[a]

Treatment	HT-29 cells (%)	Daudi cells (%)
Control	57.0 ± 3.8	92.0 ± 3.6
ovIFN-τ1mod	32.3 ± 1.1 (44)	53.8 ± 3.0 (42)
IFN-αA	12.5 ± 0.8 (78)	19.6 ± 3.3 (79)
E13R	35.0 ± 5.5 (39)	45.7 ± 2.7 (50)
K16M	23.6 ± 5.6 (59)	26.9 ± 1.0 (71)[b]
D19A	28.2 ± 6.4 (51)	36.0 ± 2.8 (61)
L24I	47.5 ± 17.4 (17)	37.9 ± 2.6 (59)
P26L	20.8 ± 6.4 (64)[b]	18.3 ± 8.3 (80)[b]
K34H	27.5 ± 7.1 (52)	34.4 ± 23.3 (63)

[a] An EC_{50} dose of ovIFN-τ1mod and equivalent molar concentration of IFN-τ mutants were added to cultures of the indicated cells and incubated for 9 (HT-29) and 3 (Daudi cells) days. Control cultures received no IFN. Results are expressed as the mean cell number $\times 10^4$ ($\times 10^2$ for Daudi cell) ± SE of three replicate experiments. % inhibition is indicated in parentheses.

[b] Significant ($P < 0.05$) increase in growth inhibition by the IFN-τ mutants relative to that of ovIFN-τ1mod.

Viability of IFN-τ Mutant-Treated Cells. As with peripheral blood lymphocytes (8), U937 cells exhibit reduced viability when treated with high concentrations of human IFN-αA, compared with comparable concentrations of ovIFN-τ1mod (Fig. 5). All of the IFN-τ mutant proteins exhibited in vitro cytotoxicity profiles similar to that of the parental ovIFN-τ1mod, which suggests that this region may not be involved in the reduced cytotoxicity of ovIFN-τ1mod.

Structural Determinations of IFN-τ Mutants. The IFN-τ mutant proteins were analyzed by CD spectroscopy to confirm that the mutations imposed did not cause gross structural changes, especially helical disruption. Because it has been established that wild-type IFN-τ and IFN-α have similar overall structures (14), IFN-τ was used as a comparative control in these studies. The observed spectrum of IFN-τ was similar to previously published results (24) and

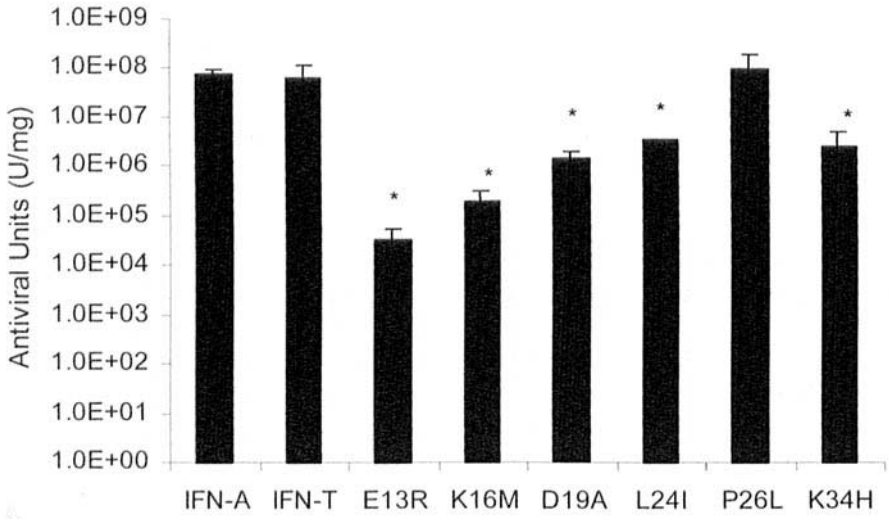

Figure 4. Antiviral activity of IFN-τ mutant proteins. Duplicate serial dilutions of each IFN were added to confluent monolayers of MDBK cells. Cells were challenged with VSV, and the cytopathic effect was assessed. Results are expressed as the mean antiviral activity in U/mg ± SE from three replicate experiments. *Significant difference determined by analysis of variance where $P < 0.05$.

Table 2. Percent Secondary Structure of IFN-τ Mutant Proteins[a]

Protein	α helix	Extended β	β Turn	Other
IFN-τ	73	1	5	20
E13R	54	13	10	22
K16M	60	6	12	22
D19A	62	7	12	19
L24I	60	7	13	20
P26L	68	7	9	14
K34H	59	8	14	19

[a] CD spectra of ovIFN-τ1mod and the IFN-τ mutants were determined by four repetitive scans using a JASCO 500C spectropolarimeter. Protein secondary structures were estimated from the CD spectral data with a root mean square error of 4% or better according to CDsstr (25).

was consistent with the X-ray structural information. Secondary structure estimates were made from the CD spectral data of IFN-τ and the IFN-τ mutants using CDsstr (25). The structural determinations derived from the spectra of the mutants indicated that the imposed mutations did cause structural changes within the molecule. The E13R mutant caused the greatest change in both the helical structure and extended β-sheet compared with wild-type IFN-τ. As would be expected, IFN-τ P26L maintained a secondary structure similar to wild-type IFN-τ.

The structural environment of each mutation was inspected using the Swiss-Prot DB Viewer's (European Bioinformatics Institute) mutation feature. The interaction between E13 and L9 was predicted to be lost in IFN-τ E13R. This could cause the orientation of the side chain in R to be flipped, enabling interaction with the negatively charged D at position 10 (data not shown). The nonconservative D19A mutation disrupted the interaction between D19 and R23 (data not shown). L or I at position 24 both extend into the solvent near helix A, and the only change in the K34H mutant was that the imidazole group was brought closer to Y at position 130 at the end of helix D (data not shown). Residue 26 is located in the first section of the AB loop. The proline-to-leucine mutation is not positioned so that it effects the helix bundle (Fig. 6). However, it is on the external surface of the structure, which suggests proximity for receptor interactions.

Discussion

There has been significant interest in finding functionally important sites on type I IFNs. In addition, there is an ongoing focus on the development of novel IFNs with unique biological activity. Our research addresses both of these areas. Many subtypes of human IFN-α exhibit different biologically specific activity (26). Consistent with this observation, IFN-τ subtypes have also recently been shown to differ in the extent of their cross-species, antiviral, and antiproliferative activities (7). In addition, ovine IFN-τ possesses unique properties that make it less cytotoxic and superior to IFN-α in sustaining the lifespan of the corpus luteum (5). It has greater therapeutic activity than does IFN-β in the treatment of EAE (10). Also, ovIFN-τ1mod has been shown to suppress papillomavirus E7 oncoprotein expression and increase p53 more effectively than human IFN-αA or the hybrid IFN-αB/D (27).

Previous structure-function studies have focused on the C terminus of ovine IFN-τ and the replacement of conserved lysines (6, 18, 19). As in the IFN-αs, the C terminus of ovine IFN-τ has been identified as being important for the antiviral and antiproliferative activity of the molecule. An 11–amino-acid C-terminal truncation and substitution at K 160 do not produce large changes in endometrial membrane receptor binding but eliminate antiviral activity and reduce antiproliferative activity on human cell lines, which suggests that this region is more important for type I IFN activities than for reproductive properties. In addition, the model for binding of type I IFNs to their common receptor proposes that residues from both the N and C termini interact with a site on IFNAR-2 (28). With this in mind, our work focused on six nonconserved residues within the N terminus. The amino-acid substitutions may affect function by affecting binding affinity of the IFN-τ mutant for its receptor by changing overall confirmation (IFN-τ K16M), affecting the local charge distribution (IFN-τ E13R, IFN-τ K16M, and IFN-τ D19A), or presenting a side chain that might interact differently with the type I IFN receptor (IFN-τ P26L).

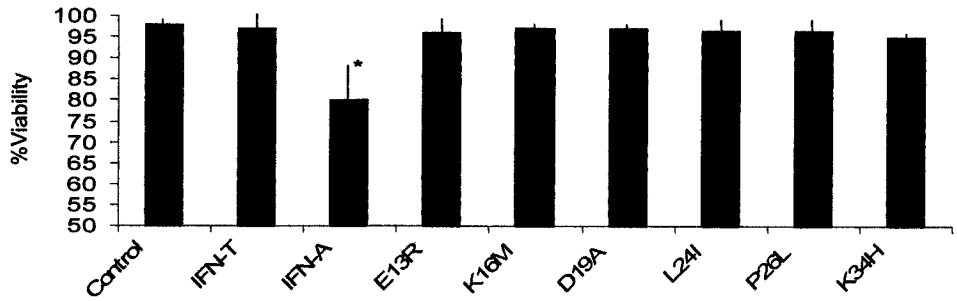

Figure 5. Viability of IFN-mutant treated cells. A total of 80,000 U/ml of ovIFN-τ1mod, IFN-αA, or IFN-τ mutants was added to 2 × 10^5 U937 cells in triplicate and incubated for 72 hrs. Control cells were treated with medium alone. Results are expressed as the mean viable cell number ± SE from three replicate experiments. *Significant difference determined by analysis of variance where $P < 0.05$.

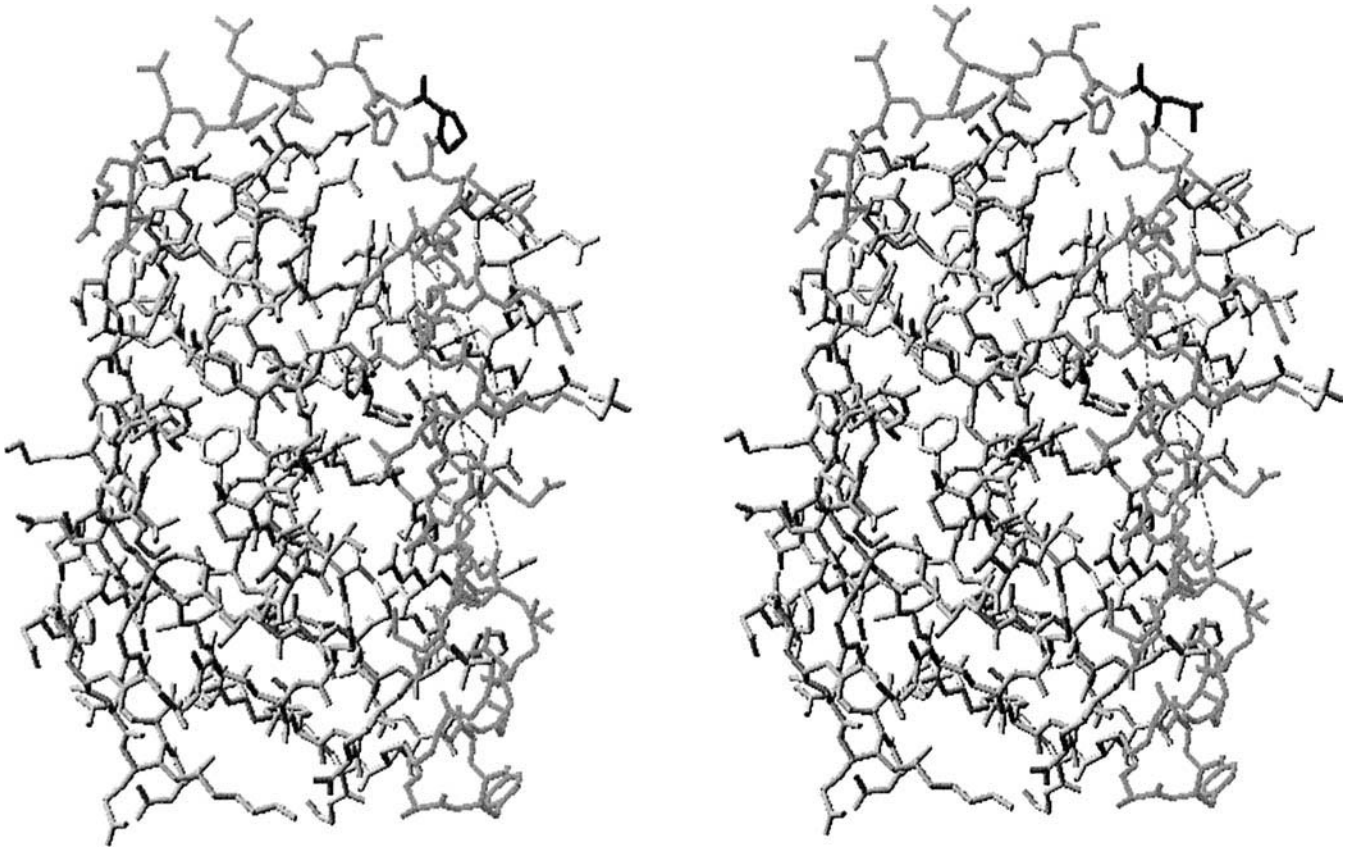

Figure 6. The three-dimensional structure of ovine IFN-τ1mod (14) was used to model mutant IFN-τ with the molecular graphics program Swiss-Prot DB Viewer. The amino-terminal residues from 1 to 34 are depicted in blue, with the remainder of the molecule in gray. The individual amino-acid substitutions are depicted in red, with the wild-type on the left and the substitution on the right.

The potency of various IFNs is reportedly related to its receptor binding affinity (29). The reduced antiproliferative potency and reduced toxicity of ovIFN-τ1mod relative to human IFN-α on MDBK cells has also been seen as a reflection of K_d, 3.90×10^{-10} and 4.45×10^{-11} for IFN-τ and IFN-α, respectively (9). However, enhanced receptor binding in our system is not always indicative of enhanced potency. This is best seen with the protein that contains the K34H mutation *versus* the P26L mutation. The K34H mutant protein shows minimal binding ability to cell-surface receptor(s) on MDBK cells, whereas the P26L mutant protein is able to bind its receptor significantly better than the native IFN-τ molecule. However, both proteins possess adequate antiviral activity on MDBK cells, with the IFN-τ P26L mutant protein possessing the highest antiviral activity. These data suggest that minimal receptor binding may lead to a signal that provides adequate antiviral activity, whereas increased binding affinity may provide maximum protection against virus. Preliminary studies in our laboratory have shown that IFN-τ is able to bind synthetic peptides that represent portions of IFNAR-2 as well as or better than peptide portions of IFNAR-1 (data not shown). Studies that assess the binding of isolated receptor chains would address the disparity seen in the present study between receptor engagement and activity. It is possible that the minimal binding seen with IFN-τ K34H represents specific binding to IFNAR1 or IFNAR2, which is sufficient to generate a signal.

Effects of the N-terminal substitutions on the antiproliferative activity of various carcinoma cell lines were cell type specific, but each of the mutants maintained equivalent antiproliferative activity relative to the parental IFN-τ on at least one cell line. Thus, antiproliferative and antiviral activity are dissociated in this system. This is consistent with differential effects on antiviral and antiproliferative activities of antibodies to a conserved IFN-α peptide (30). A comparison of cell lines that were either sensitive or resistant to IFN-α–mediated growth inhibition suggested that the differential effects are due to the role of the MAPK pathway in IFN-α–mediated growth inhibition but not in inducing an antiviral state (31). The ability of IFN-τ to induce gene expression has recently been attributed to the activation of p38 pathway (32). The P26L mutation appears to be the most interesting of this panel of variant proteins, and the L is only found in a few IFN-α subtypes, including IFN-αA, the subtype used for our comparisons. The antiviral activity of IFN-τ P26L on MDBK cells was equivalent to that of the wild type. Of interest, IFN-α4

mutated at this same position showed a slight increase in antiviral activity when tested on MDBK cells (33). This mutation exhibited improved antiproliferative activity over that of ovIFN-τ1mod in two of the three cell lines tested, with activity on Daudi cells superior to that of the human IFN-α. Finally, it retained the reduced *in vitro* cytotoxicity seen with the ovIFN-τ1mod. Therefore, the IFN-τ P26L mutant might have advantages for treatment of viral and neoplastic diseases.

The single-amino-acid substitutions within the N terminus of ovIFN-τ1mod did not affect either cross-species activity or the subtype-specific reduction in cytotoxicity. This suggests that either multiple mutations are required to affect these properties or the cross species activity and lower toxicity of ovIFN-τ1mod may be conferred by another structural domain of the molecule. For example, residues in helix C have been thought to bind to a site on IFNAR-1 (28). In addition, substitutions in helix C of human IFN-β increased its cross-species activity and eliminated its ability to associate tyrosine-phosphorylated IFNAR-1 and IFNAR-2, whereas point mutations in the AB loop had no effect on either property (16). The determination of the relationship between sequence and structural elements of the type I IFNs will facilitate the rational design of molecules with therapeutic potential.

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1. Pestka S, Langer JA, Zoon KC, Samuel CE. Interferons and their actions. Annu Rev Biochem 56:727–777, 1987.
2. Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW. Ovine interferon-τ inhibits estrogen receptor up-regulation and estrogen-induced luteolysis in cyclic ewes. Endocrinology 136:4932–5944, 1995.
3. Bazer FW, Spencer TE, Ott TL. Placental interferons. Am J Reprod Immunol 35:297–308, 1996.
4. Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. Nature 330:377–379, 1987.
5. Roberts RM, Cross JC, Leaman DW. Interferons as hormones of pregnancy. Endocr Rev 13:432–452, 1992.
6. Alexenko AP, Leaman DW, Li J, Roberts RM. The antiproliferative and antiviral activities of IFN-τ variants in human cells. J Interferon Cytokine Res 17:769–779, 1997.
7. Alexenko AP, Ealy AD, Roberts RM. The cross-species antiviral activities of different IFN-τ subtypes on bovine, murine, and human cells: contradictory evidence for therapeutic potential. J Interferon Cytokine Res 19:1335–1341, 1999.
8. Pontzer CH, Yamamoto JK, Bazer FW, Ott TL, Johnson HM. Potent anti-feline immunodeficiency virus and anti-human immunodeficiency virus effect of IFN-τ. J Immunol 158:4351–4357, 1997.
9. Subramaniam PS, Khan SA, Pontzer CH, Johnson HM. Differential recognition of the type I interferon receptor by interferons τ and α is responsible for their disparate cytotoxicities. Proc Natl Acad Sci U S A 92:12270–12274, 1995.
10. Soos JM, Subramaniam PS, Hobeika AC, Schiffenbauer J, Johnson HM. The IFN pregnancy recognition hormone IFN-τ blocks both development and superantigen reactivation of experimental allergic encephalomyelitis without associated toxicity. J Immunol 155:2747–2753, 1995.
11. Radhakrishnan R, Walter LJ, Hruza A, Reichert P, Trotta PP, Nagabhushan TL, Walter MR. Zinc mediated dimer of human interferon-α 2b revealed by X-ray crystallography. Structure 4:1453–1463, 1996.
12. Senda T, Saitoh S, Mitsui Y. Refined crystal structure of recombinant murine interferon-β at 2.15 A resolution. J Mol Biol 253:187–207, 1995.
13. Karpusas M, Nolte M, Benton CB, Meier W, Lipscomb WN, Goelz S. The crystal structure of human interferon β at 2.2-A resolution. Proc Natl Acad Sci U S A 94:11813–11818, 1997.
14. Radhakrishnan R, Walter LJ, Subramaniam PS, Johnson HM, Walter MR. Crystal structure of ovine interferon-τ at 2.1 A resolution. J Mol Biol 286:151–162, 1999.
15. Mitsui Y, Senda T, Shimazu T, Matsuda S, Utsumi J. Structural, functional and evolutionary implications of the three-dimensional crystal structure of murine interferon-β. Pharmacol Ther 58:93–132, 1993.
16. Runkel L, Pfeffer L, Lewerenz M, Monneron D, Yang CH, Murti A, Pellegrini S, Goelz S, Uze G, Mogensen K. Differences in activity between α and β type I interferons explored by mutational analysis. J Biol Chem 273:8003–8008, 1998.
17. Pontzer CH, Ott TL, Bazer FW, Johnson HM. Localization of an antiviral site on the pregnancy recognition hormone, ovine trophoblast protein 1. Proc Natl Acad Sci U S A 87:5945–5949, 1990.
18. Li J, Roberts RM. Interferon-τ and interferon-α interact with the same receptors in bovine endometrium: use of a readily iodinatable form of recombinant interferon-τ for binding studies. J Biol Chem 269:13544–13550, 1994.
19. Li J, Roberts RM. Structure-function relationships in the interferon-τ (IFN-τ). Changes in receptor binding and in antiviral and antiproliferative activities resulting from site-directed mutagenesis performed near the carboxyl terminus. J Biol Chem 269:24826–24833, 1994.
20. Van Heeke G, Ott TL, Strauss A, Ammaturo D, Bazer FW. High yield expression and secretion of the ovine pregnancy recognition hormone interferon-τ by Pichia pastoris. J Interferon Cytokine Res 16:119–126, 1996.
21. Swann SL, Bazer FW, Villarete LH, Chung A, Pontzer CH. Functional characterization of monoclonal antibodies to interferon-tau. Hybridoma 18:399–405, 1999.
22. Pontzer CH, Johnson HM. Measurement of interferons. Methods Neurosci 24:3–9, 1995.
23. Forthofer RN. Introduction to Biostatistics: A guide to design, analysis, and discovery. San Diego: Academic Press, pp567, 1995.
24. Jarpe MA, Johnson HM, Bazer FW, Ott TL, Curto EV, Krishna NR, Pontzer CH. Predicted structural motif of IFN τ. Protein Eng 7:863–867, 1994.
25. Johnson W. Analyzing protein circular dichroism spectra for accurate secondary structures. Proteins 35:307–312, 1999.
26. Zoon KC, Miller D, Bekisz J, zur Nedden D, Enterline JC, Nguyen NY, Hu RQ. Purification and characterization of multiple components of human lymphoblastoid interferon-α. J Biol Chem 267:15210–15216, 1992.
27. Johnson JA, Hochkeppel HK, Gangemi JD. IFN-τ exhibits potent suppression of human papillomavirus E6/E7 oncoprotein expression. J Interferon Cytokine Res 19:1107–1116, 1999.

28. Mogensen KE, Lewerenz M, Reboul J, Lutfalla G, Uze G. The type I interferon receptor: structure, function, and evolution of a family business. J Interferon Cytokine Res 19:1069–1098, 1999.
29. Aguet M, Grobke M, Dreiding P. Various human interferon α subclasses cross-react with common receptors: their binding affinities correlate with their specific biological activities. Virology 132:211–216, 1984.
30. Barasoain I, Portoles A, Aramburu JF, Rojo JM. Antibodies against a peptide representative of a conserved region of human IFN-α: differential effects on the antiviral and antiproliferative effects of IFN. J Immunol 143:507–512, 1989.
31. Arora T, Floyd-Smith G, Espy MJ, Jelinek DF. Dissociation between IFN-α–induced anti-viral and growth signaling pathways. J Immunol 162:3289–3297, 1999.
32. Doualla-Bell F, Koromilas AE. Induction of PG G/H synthase-2 in bovine myometrial cells by interferon-τ requires the activation of the p38 MAPK pathway. Endocrinology 142:5107–5115, 2001.
33. Waine GJ, Tymms MJ, Brandt ER, Cheetham BF, Linnane AW. Structure-function study of the region encompassing residues 26–40 of human interferon-α 4: identification of residues important for antiviral and antiproliferative activities. J Interferon Res 12:43–48, 1992.