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Differences in Activity between α and β Type I Interferons Explored by Mutational Analysis*

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Type I interferon (IFN) subtypes α and β share a common multicomponent, cell surface receptor and elicit a similar range of biological responses, including antiviral, antiproliferative, and immunomodulatory activities. However, α and β IFNs exhibit key differences in several biological properties. For example, IFN- β , but not IFN- α , induces the association of tyrosine-phosphorylated receptor components ifnar1 and ifnar2, and has activity in cells lacking the IFN receptor-associated, Janus kinase tyk2. To define the structural basis for these functional differences we produced human IFN- β with point mutations and compared them to wild-type IFN- β in assays that distinguish α and β IFN subtypes. IFN- β mutants with charged residues (N86K, N86E, or Y92D) introduced at two positions in the C helix lost the ability to induce the association of tyrosine-phosphorylated receptor chains and had reduced activity on tyk2-deficient cells. The combination of negatively charged residues N86E and Y92D (homologous with IFN- α 8) increased the cross-species activity of the mutant IFN- β s on bovine cells to a level comparable to that of human IFN- α s. In contrast, point mutations in the AB loop and D helix had no significant effect on these subtype-specific activities. A subset of these latter mutations did, however, reduce activity in a manner analogous to IFN- α mutations. The effects of these mutations on IFN- β activity are discussed in the context of a family of related ligands acting through a common receptor and signaling pathway.

The mammalian type I IFNs,¹ produced in response to viral infection and other inducers, are divided into α and β subtypes on the basis of their reactivity with antisera raised against IFNs derived, respectively, from leukocytes and fibroblasts (1). The human IFN- α s are encoded by a family of at least 15 different genes, while IFN- β is the unique member of its subtype (2). Primary sequence comparison between the α and β subtypes reveal an approximately 50% amino acid homology, while the amino acid homologies between the IFN- α subtypes are approximately 80% (2, 3), reinforcing the division between

IFN- α and - β subtypes.

As the pleiotropic nature of these cytokines became apparent, with both subtypes eliciting a similar range of biological activities (3), differences between α subtypes, and between IFN- α and - β s, in potency and cell type specific activities were noted (4). In particular, IFN- β elicits a markedly higher antiproliferation response in some cell types such as (5), embryonal carcinoma, melanoma and melanocytes than do IFN- α s (6, 7, and references therein). Higher potency of IFN- β in treatment of multiple sclerosis and certain cancers has been observed (7).

The entire class of type I IFNs elicit their biological activities through engagement of a common cell surface receptor (8–10). Two chains of the receptor, ifnar1 and ifnar2, both members of the type two cytokine receptor family, have been identified (11–15). Both components are necessary for function and in the absence of either there is neither high affinity binding nor biological effect (14, 16, 17). The intracellular portions of the receptor subunits are bound by tyrosine kinases, jak1 (12, 18) and tyk2 (19, 20), members of the Janus kinase family. Upon ligand binding these kinases are activated and phosphorylate members of the STAT family of transcription factors (21), as well as ifnar1 and 2. A further property that distinguishes these IFN subtypes is that IFN- β , but not IFN- α , induces association of tyrosine phosphorylated ifnar1 and 2, detectable by precipitation with anti-ifnar1 antibodies (22–25). In addition, tyk2-deficient cells retain partial responsiveness to IFN- β , but are completely unresponsive to IFN- α s (26). Complementation of the tyk2 deficiency by expression of a kinase-inactive tyk2 partially restores IFN- α 8 binding and activity, but has no effect on the IFN- β binding to these cells although it augments IFN- β -induced signaling (27). Thus, potency and specificity differences between IFN- α s and - β s may reflect differences in receptor interaction.

The type I IFNs are closely related members of the helical cytokine family (28). Resolution of the three-dimensional structures for crystals of murine IFN- β (29, 30) and human IFN- α 2b (31) revealed that their overall structure is very similar, and that these IFNs are composed of 5 helices joined by loops of various lengths. Inspection of the crystal structures in light of previous extensive IFN- α mutational analyses (reviewed in Refs. 15 and 32) allows identification of putative domains likely to be involved in receptor interactions. Examination of structural models in regions of mutational hotspots, such as the AB loop, D helix, DE loop (32), and the C helix (33, 34), directed us to exposed residues available for receptor binding.

In this study we describe the functional consequences of site-directed mutations of human IFN- β . Our results reveal the importance of C helix residues in conferring subtype specific activities on IFN- β as judged from activity and biochemical assays. Mutation of AB loop (Arg²⁷ and Arg³⁵) and D helix

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¹ The abbreviations used are: IFN, interferon; HAT, hypoxanthine, aminopterin, and thymidine; 6TG, 6-thioguanine; MDBK, Madin-Darby bovine kidney; tyk, tyrosine kinase; STAT, signal transducers and activators of transcription.

(Lys¹²³, but not Arg¹²⁴) residues reduces activity in all assays and are qualitatively similar to effects seen for homologous IFN- α mutations. However, the AB loop and D helix IFN- β mutants retain IFN- β -specific activities in assays that distinguish between IFN- α and - β subtypes.

EXPERIMENTAL PROCEDURES

Notation for IFN Amino Acid Sequences—We use the single letter code for amino acids. Substitutions are given as X_nY , where X is the amino acid replaced, n , its number in the sequence and Y , the replacement. To facilitate discussion, we number the IFN- α amino acid sequences from the NH₂ terminus according to the convention that counts the deletion at position 44 of IFN- α 2 (29, 30, 32). Human IFN- β is numbered from the NH₂ terminus without deletion. This alignment leaves all α positions, n , with homologous β positions at $n + 2$, up to the COOH terminus of IFN- β . The five α helices of IFN are labeled from the NH₂ terminus: ABCDE, the loops are labeled with the letters of the helices at their NH₂-terminal and COOH-terminal ends.

Construction, Production, and Evaluation of Mutant IFNs—Substitutions introduced into human IFN- β are given in Fig. 1. The mutations were introduced into the human IFN- β gene carried on plasmid pJC017, a COS cell expression vector (Biogen, Inc. Cambridge, MA), using the transformer site-directed mutagenesis kit from CLONTECH Laboratories, Inc. (Palo Alto, CA). COS 7 cells were transiently transfected, and the supernatants from cultures (3 days posttransfection) were screened for the presence of immunoreactive IFN- β and then for biological activity on human HL116 cells, which carry a luciferase reporter under control of an IFN- α - β -inducible promoter (33). Quantitation of IFN expression levels was performed by enzyme-linked immunosorbent assay utilizing rabbit polyclonal anti-IFN- β 1a serum to coat plates, biotinylated rabbit polyclonal antibodies as a secondary reagent, followed by streptavidin-coupled horseradish peroxidase (Jackson Immunochemical, West Grove, PA). Enzyme-linked immunosorbent assay values were confirmed by Western blot analyses using a second rabbit anti-IFN- β 1a polyclonal serum. Western blotting revealed two interferon bands corresponding to unglycosylated and glycosylated forms of the molecule which were present in roughly equal proportions. Molarities of interferons based on biological activity referenced to fully glycosylated, purified IFN- β 1a were always less than those estimated by enzyme-linked immunosorbent assay. Experiments to investigate the effect of glycosylation on IFN- β activity suggest a 2-fold lower specific activity of unglycosylated versus glycosylated forms (53). All the results presented in this report compare mutant and wild-type preparations that had similar proportions of glycosylated interferon. Supernatants were assayed for activity on cells with luciferase as an IFN-inducible reporter. Each production batch included a transfection with cDNA for unmutated wild-type IFN- β and a mock transfection control. Preliminary studies had shown that IFN- β could be routinely produced with supernatants yielding in a range around 1.0 nM and that such supernatants, stored at 4 °C, retained their activity over several months.

Substitutions in the C helix of IFN- α were obtained by site-directed mutations introduced into the cDNA encoding the IFN hybrid α 8[60] α 1[92] α 8 (35). This hybrid, in which part of the B helix, the BC loop, and part of the C helix are derived from human IFN- α 1, is labeled P. Construction, expression, purification, and characterization of the P mutants have been described in detail elsewhere (33).

Immunoprecipitations—Receptor chain coimmunoprecipitations were performed essentially as described previously (24). For each experimental point 2×10^8 human Daudi B cells (ATCC no. CCL 213) were treated with wild-type or mutant IFN- β (2,500 units/mL, 10 min, 37 °C). Receptor proteins were immunoprecipitated from extracts using either anti-*ifnar1* monoclonal antibody (AA3, Biogen Inc.), anti-*ifnar2* monoclonal antibody (AB.B7.2, Biogen Inc.), or control mouse sera. Immune complexes were analyzed by Western blotting of SDS-polyacrylamide gel electrophoresis, 7.5% gels, probed with anti-phosphotyrosine monoclonal AB-2 antibodies (Oncogene Research Products, Cambridge, MA) followed by anti-mouse IgG coupled with horseradish peroxidase (Amersham Corp.). Blots were developed using enhanced chemiluminescence (ECL, Amersham). The blots were stripped and reblotted with anti-*ifnar1* monoclonal antibody to verify that equal amounts of protein were loaded in each lane.

Cells and IFN Assays—The recombinant IFNs, human IFN- β was from Biogen Inc.; human IFN- α 2 (2c) was a gift from Dr G. Adolf, Ernst Boehringer Institute, Vienna, Austria; human IFN- α 8 and - α 1 and hybrids of these were a gift from Ciba-Geigy, Basel, Switzerland. Concentrations of the IFNs were estimated against IFN reference prepara-

tions, pure recombinant IFNs, IFN- α at 5.0 nM, and IFN- β at 250 nM of active monomer. The IFN- α reference at 5 nM corresponds to 20,000 IU (MRC 69/19). Cell sensitivity to IFN was estimated in terms of the mean concentration required to obtain a 50% response in any given assay (see Table I).

The derivation and construction of the human HL116 cell line, carrying the luciferase gene under control of the IFN-inducible 6–16 promoter, and its use in the assay of IFNs has been described in detail elsewhere (33). End points were at 50% luciferase induction and assays were repeated to obtain a precision (95%) of approximately ± 0.3 log titer. Where activities are presented as percent of the unmutated form, this corresponds to a precision of 50–200%. Relative activities of mutant IFNs, estimated by luciferase induction, were confirmed at least once by both antiviral and antiproliferative assay.

The human 2fTGH-derived, 11.1 (*tyk2*-deficient), cells do not express the intracellular tyrosine kinase *tyk2* (26). They are also *hprt*⁻ (hypoxanthine-phosphoribosyl transferase) and carry bacterial *gpt* (xanthine/guanine-phosphoribosyl transferase) linked to the IFN sensitive 6–16 promoter, allowing IFN to be assayed in terms of its capacity to induce cellular growth in hypoxanthine, aminopterin, and thymidine (HAT) containing medium or cell mortality in medium containing 6-thioguanine (6TG) (27, 36). Strain A27 of the 11.1 *tyk2*-deficient cells was obtained from a fast growing colony of 11.1 cells, cultivated in HAT plus 1.0 nM IFN- β , which was then cloned and reselected in HAT plus 50 pM IFN- β and recloned. All selectant cultures, and clones derived from them, were tested for their sensitivity toward HAT alone and cultured in 6TG alone. The strain A27 died in HAT, grew in 6TG alone, and showed a growth response (positive in HAT, negative in 6TG) in the presence of 10 pM IFN- β , in contrast to the 11.1 cells which were between 10- and 100-fold less sensitive in their growth responses to IFN- β . Like the 11.1 cells, A27 produced no detectable immunoreactive *tyk2* (20). Further selections from A27 produced only clones that either grew in HAT alone and/or were unable to grow in 6TG alone. The characteristics of A27 have remained stable. The enhanced sensitivity of A27 to IFN, also seen in antiviral assay, allowed a full dose response range for the biological assay of the mutant IFN- β s.

For IFN antiviral assays, bovine MDBK (ATCC no. 6071), primary equine dermis NBL6 (ATCC no. CCL57) at passage 20–24, human amnion-derived WISH and *tyk2*⁻ cells were treated overnight with different concentrations of IFN and then challenged with vesicular stomatitis virus at the optimal multiplicities of infection, 0.01, 0.001, 0.001, and 0.01 for the respective cell lines. When control cultures showed 100% cell destruction, an index estimating the cytopathic effect of the virus was scored as a function of IFN concentration, with each point the mean of six replicates. End points were at 50% protection and assays were repeated to obtain a precision (95%) of approximately ± 0.3 log titer. Where antiviral activities of IFNs are presented as percent of the unmutated form, this corresponds to a precision of 50–200%. The sensitivity of equine NBL6 cells toward IFN- β is such that mutant IFN- β preparations with reduced activity fail to give an end point on antiviral titration; such mutants are scored <wild type. The antiproliferative effects of the mutant IFNs were assayed on the Burkitt lymphoma-derived Daudi cell line.

Modeling and Assessment of Side Chain Accessibility—Models of IFN- α and IFN- β were generated by homology modeling with the Modeller, Protein Design, and Protein Health functions available with the Quanta package (MSI Inc., San Diego CA), using the α carbon coordinates of murine IFN- β (Brookhaven accession no. 2RMI) as template. Solvent accessibility of residues and their side chains and were obtained with the default settings of the Protein Design function of Quanta. Inspection of the recently published figures for IFN- α 2b (31) and comparison with the unpublished coordinates for human IFN- β (37) suggest that these models were useful for the α helices and for the AB loop, COOH-terminal to cysteine 29 (cysteine 31 in human β); less so for the positioning of residues in the other loops.

RESULTS

Design of IFN- β Mutants—The putative distribution of α helices (A through E) and loops (identified by their bounding helices) are shown in the primary sequence comparison shown in Fig. 1. The mutations in the C helix of IFN- β were confined to residues 86 and 92 (homologous with 84 and 90 in the IFN- α s) and were concerned with substituting the noncharged side chains with residues that would be charged at neutral pH. Previous work had demonstrated that these residues participate to confer subtype α 8-specific activities onto a hybrid

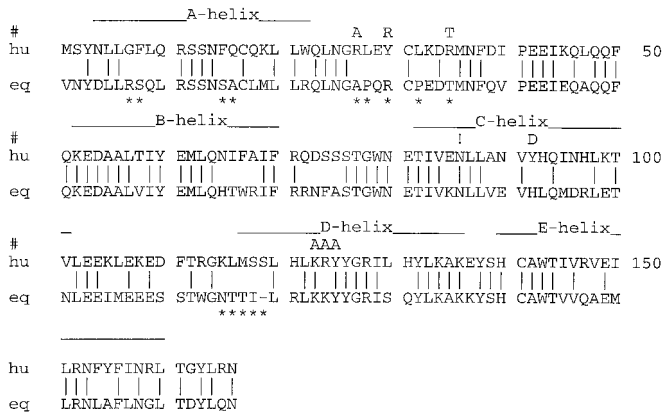


FIG. 1. Point mutations in human IFN-β. Sequence alignment of human (*hu*) and equine (*eq*) IFN-βs, showing substitutions introduced into the former. Vertical bars, identities; #, amino acid substitutions in human IFN-β (!, N86: K or E); *, groupings in equine IFN-β having distinct differences from the human with respect to size and hydrophobicity of amino acid side chains; —, position of α helices in murine IFN-β (30, 31, 37)

IFN-α1 (33), implicating them as important residues for defining the character of receptor mediated, subtype-specific activities. Single mutations N86E, N86K, and Y92D, as well as doubly substituted mutants at these positions, N86E,Y92D and N86K,Y92D, were investigated in this study.

Sequence comparisons between type I IFN subtypes of equine (38) and human origin revealed distinctly different amino acids in certain regions of the molecules, including the A helix, NH₂-terminal AB loop, and D helix (Fig. 1). Mutational analysis of IFN-αs have identified the NH₂-terminal AB loop as a critical domain for activity and receptor binding (reviewed in Refs. 15 and 32). The IFN-αs of equine and human origin are conserved in this region and retain a high degree of cross-species reactivity. These observations suggested that IFN-α and -β functional differences may reside in amino acid differences of the AB loop. Therefore, we chose substitutions of human IFN-β residues Arg²⁷, Tyr³⁰, and Arg³⁵ with homologous residues of equine IFN-β.

The D helix was scanned by alanine substitutions at residues Lys¹²³, Arg¹²⁴ (32), and Tyr¹²⁵ (39), that had previously been shown to be critical for IFN-α function and, therefore, likely to be involved in receptor interactions (15).

All residues mutated, except Y125A, were estimated to be at least partially exposed to solvent. These predictions have been confirmed on the recently solved, three-dimensional structure of the human IFN-β1a (37). The likelihood that Tyr¹²⁵ is a buried residue, involved in intramolecular bonds, was confirmed on the three-dimensional structure of human IFN-β1a (37).

Three biological assays which distinguish between α and β IFNs are shown in Table I, where relative potencies of the two type I IFNs are also given. Typically, as for WISH cells or HL116 cells assayed with encephalomyocardial virus, the relative potencies of α and β IFNs are approximately the same on human cell lines. However, on equine NBL6 cells IFN-α has 2000 times more antiviral activity than does IFN-β. On bovine MDBK cells IFN-α has 15 times more antiviral activity than does IFN-β. On the human tyk2⁻ line 11.1 (strain A27) IFN-β has at least 1000-fold greater antiviral activity than does IFN-α (19, 20, 26, 36). In addition, to gain insights into effects of mutations on IFN interactions with its receptor, the mutant IFN-βs were analyzed in a biochemical assay which detects the capacity of IFN-β, but not IFN-α, to induce a co-immunoprecipitable complex between tyrosine phosphorylated ifnar1 and ifnar2 (22, 23, 24, 25).

TABLE I
Differences in activities induced by human α and β IFNs in the various assay systems used

Assay system induced activity ^a	Level of responsiveness		β/α difference
	IFN-β	IFN-α	
Equine cells, antiviral	~2 nM	~1 pM	2000
Bovine MDBK cells	~3 pM	~0.2 pM	15
Human WISH cells, antiviral	(0.6–15)	(0.03–0.9)	0.4
Human tyk2 ⁻ cells, antiviral	~0.4 pM	~1.0 pM	<0.001
Human Daudi cells (ifnar1/ifnar2 co-IP) ^b	~100 pM	>100 nM	Yes No

^a Antiviral activities expressed as average (~) concentrations giving a 50% response; fiducial limits at 95% in parentheses.

^b Ligand-induced coimmunoprecipitation (co-IP) of phosphorylated ifnar1 and ifnar2.

The relative potencies of the IFN-β mutants that we determined in these assays are shown in Table II and are expressed as a percentage of wild-type IFN-β activity. We have used human HL116 cells, which carry a luciferase reporter gene under control of an IFN-inducible promoter, as the reference assay for human IFNs (33). Relative activities of mutant IFNs, estimated by luciferase induction, were confirmed by both antiviral assay on WISH cells and by antiproliferative assay on Daudi cells. All mutations at solvent exposed residues were found to result in an activity comparable to wild type in at least one of the assay systems used, suggesting that when functional differences were detected that they indeed reflect involvement of the mutated side chain. Expression of the Y125A mutant could not be detected at wild-type levels using two polyclonal sera, suggesting that either the substitution had collapsed a major epitope or that mutant protein could not be stably produced.

Substitutions in the C Helix—Substitution of the charged residues at IFN-β positions 86 and 92, either individually (N86E, N86K, and Y92D) or together (N86E,Y92D and N86K,Y92D), did not alter the specific activity as measured by luciferase induction on human HL116 cells, antiviral potency on equine NBL6 cells (Table II), or antiproliferative activity on human Daudi B cells (data not shown). However, in several assays where IFN-α and -β properties are distinctly different, the double substitutions were shown to result in biological activities different from wild-type IFN-β.

Table II shows the relative specific activities of IFN-β mutants on tyk2-deficient cells. The C helix double mutants showed a reduction in specific activity on these cells, suggesting a more IFN-α-like activity of these double mutants. While these differences (3-fold for N86E,Y92D and 2-fold for N86K,Y92D) were relatively small, they were seen consistently with different production batches. The tyk2-deficient cells carry an IFN-inducible construct that permits a bypass of an aminopterin block on purine synthesis (36). Consequently, IFN can be assayed for its capacity to induce cellular growth in HAT-containing medium or cell mortality in medium containing 6TG. Using this assay we confirmed that the C helix double mutants, N86E,Y92D and N86K,Y92D, were reduced in their activity on tyk2-deficient cells. Mutations in other regions of IFN-β did not alter activities on tyk2-deficient cells with the exception of Y30R, which reproducibly showed a slightly increased activity (2–3-fold) on these cells (Table II).

The mutants were analyzed on human Daudi Burkitt's lymphoma cells for their capacity to induce the IFN-β-specific association of tyrosine-phosphorylated ifnar1 and ifnar2 chains, detectable following immunoprecipitations with anti-ifnar1 antibodies. Fig. 2B shows that all mutants, except the

double C helix mutants (N86E,Y92D and N86K,Y92D) retained the IFN- β -specific capacity to induce association of tyrosine-phosphorylated receptor chains. It is important to note that, although C helix double mutants failed to induce this association, those mutants, as well as IFN- α (22, 23, 24), stimulate the tyrosine phosphorylation of both receptor chains, as shown in anti-ifnar2 (Fig. 2A) or anti-ifnar1 immunoprecipitates (Fig. 2, B1–3). The results from three different anti-ifnar1 coimmunoprecipitation assays are shown in panels B1–3 (Fig. 2).

Table II shows that all C helix mutants, whether single or double, exhibit enhanced antiviral potencies on bovine cells when compared with wild-type IFN- β , while mutations in the AB loop and D helix had no effect on this cross-species activity. Fig. 3 compares the effect of C helix substitutions on the antiviral activity of IFN- β in bovine MDBK cells with that of similar substitutions in IFN- α . The IFN- α s and their derivatives were all uniformly active on bovine cells, which are 15-fold more sensitive to human IFN- α s than IFN- β . The chimeric IFN- α , labeled *P* in Fig. 3, was constructed from α 8, with the

relevant part of the C helix derived from α 1 (point mutations at α 1 sequences are K84E,Y90D). This chimeric IFN- α was used in previous studies to demonstrate that these residues participate to confer subtype specific activity on the chimera (33). Fig. 3 shows that in contrast to IFN- α the specific activity of IFN- β is increased by introduction of negatively charged side chains of low pK at positions 86 and 92; thus, in descending order of IFN- β activity on bovine cells: E > N > K at position 86, and D > Y at position 92. The cross-species activity of C helix IFN- β mutants N86E,Y92D, N86E, and Y92D on bovine cells was increased to levels comparable to that of human IFN- α s.

These data demonstrate that C helix residues 86 and 92 contribute to activities distinctive for IFN- β and implicate early events in receptor engagement as key to initiating subtype specific signals. In addition, the cross-species activity of IFN- β mutants on bovine cells was altered by introduction of charged residues in the C helix.

AB Loop and D Helix Mutations—The substitutions R27A, Y30R, and R35T in the AB loop were made to mimic analogous residues of the equine IFN- β (Fig. 1). None of the mutant IFN- β s showed higher activity on equine cells than wild-type IFN- β (Table II), as would have been expected if these residues were an important site for equine receptor recognition. Substitution Y30R increased by nearly 3-fold the IFN- β activity on tyk2-deficient cells, but had neither effect on activity in bovine antiviral assays nor on the IFN- β -induced association of receptor chains (Table II). Mutations at two positions in the AB loop, R27A and R35T, caused a diminution in activity in assays on bovine and human cells. Residue Arg²⁷ is not conserved between IFN- α and - β subtypes, while Arg³⁵ is conserved in all human IFN- α s and - β s (2, 3). The IFN- α homologue Arg³³ is particularly sensitive to mutational change, where even the charge conserved mutation R33K produces more than 100-fold loss in activity (33). The mutation R35T in IFN- β produced a modest 10–30-fold loss in antiviral potency in all assay systems, except on equine cells where it assayed as wild-type activity.

The D helix was scanned by alanine substitutions at residues Lys¹²³ and Arg¹²⁴, which were previously shown to be sensitive to mutation in IFN- α s (32). Tandem charge reversal mutations in IFN- α s of the basic residue pair (Lys¹²¹, Arg¹²¹, or Arg¹²²) produces a 3-log loss in activity, while apolar (leucine substitution) mutations resulted in a 1–2-log loss in antiviral activity (32). The individual substitution K123A in IFN- β produced a

TABLE II
Specific activities of mutant human β IFNs compared to the unmutated form (*wt*) in the assay systems described in Table 1

IFN	Cell type for assay of specific activity as % of <i>wt</i> ^a IFN- β			
	Human HI116Lu ^b	Human tyk2 ^{-/-}	Bovine MDBK	Equine NBL6
	%			
<i>wt</i>	100	100	100	100
AB loop				
R27A	30	10	≤100 ^c	< <i>wt</i>
Y30R	100	280	100	100
R35T	10	< <i>wt</i>	3	100
C helix				
N86E	100	100	700	100
N86K	100	100	50	100
Y92D	100	≤100 ^c	450	100
N86E,Y92D	100	30	1200	100
N86K,Y92D	100	50	180	100
D helix				
K123A	40	< <i>wt</i>	20	100
K124A	100	100	100	< <i>wt</i>
Y125A ^d				

^a Wild type: supernatants from COS cells transfected with cDNA for unmutated IFN- β . <*wt* means that no end point could be scored.

^b Lu assay as concentration inducing 50% luciferase. Assays on other cells: concentration giving 50% antiviral protection.

^c At least one batch was clearly less while the others were equal to *wt*.

^d Transfections of COS cells failed to produce immunoreactive (enzyme-linked immunosorbent assay and Western blotting) material.

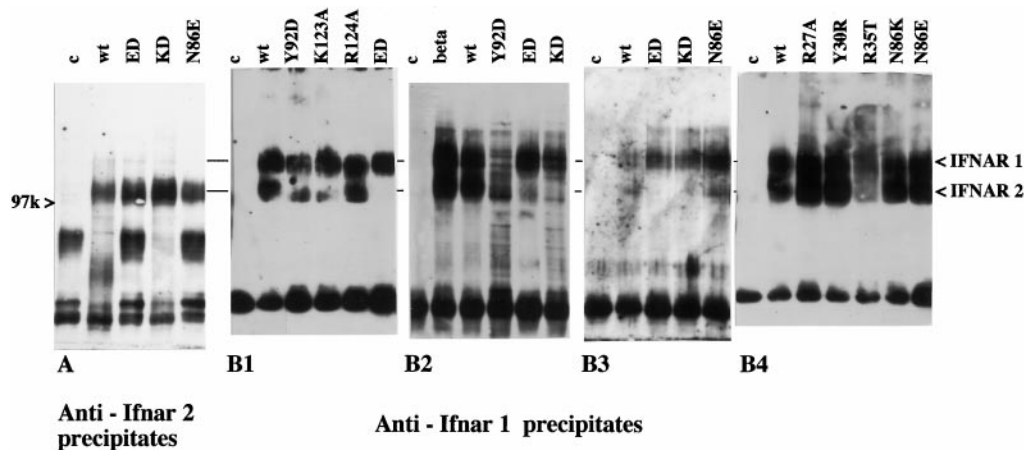


FIG. 2. Western blot analysis with anti-phosphotyrosine of immunoprecipitates from extracts of Daudi Burkitt's lymphoma cells treated with mutant IFN- β s. A, immunoprecipitation with monoclonal antibody specific for the extracellular domain of ifnar2. B, immunoprecipitation with monoclonal antibody specific for the extracellular domain of ifnar1. Replicates are from different production batches. Wild-type (*wt*) and mutant IFNs were supernatants from COS cells transfected with the appropriate constructs; *c*, conditioned medium from mock transfected cells; *beta*, the purified reference IFN- β . Arrowheads indicate the position of receptor proteins; 97k is the size of the closest migration marker.

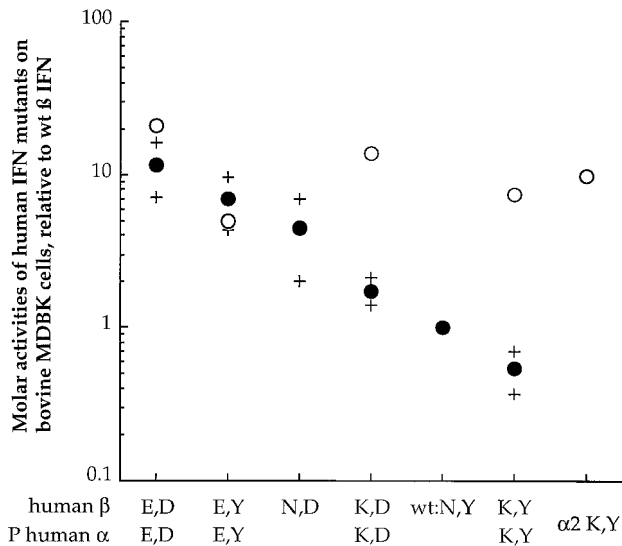


FIG. 3. The effect of substitutions at residues in the C helix of human α (positions 84 and 90) and human IFN- β (positions 86 and 92) on the antiviral activity of these subtypes on bovine MDBK cells. ○, IFN- α ; ●, IFN- β . Wild-type (*wt*) IFN- β is taken as unit reference for each transfection batch; +, standard error on means from different batches of the IFN- β s. A relative specific activity of 1.0 corresponds to a 50% end point at 3.0 pM, see Table I. *P* is the human $\alpha 8$ hybrid IFN, with part of the C helix from $\alpha 1$ (Lys⁸⁴ and Tyr⁹⁰), into which the C helix residues from $\alpha 8$ (Glu⁸⁴ and Asp⁹⁰) were introduced; $\alpha 2$ is the 5.0 nM reference (see “Experimental Procedures”). The IFNs $\alpha 1$ and $\alpha 8$ are not shown but assayed within the range of the IFN- α mutants.

modest 3-fold decrease in antiviral activity, while R124A had no effect on activity of IFN- β assayed on either human or bovine cells. The importance of these residues appears to be reversed on equine cells, where K123A was equivalent to wild type in antiviral potency, while R124A fails to induce any antiviral protection even when assayed at twice the wild-type IFN- β concentration.

Substitutions of AB loop and D helix residues did not markedly alter IFN- β activities in any of the assays that distinguish α and β subtypes. The mutation of two highly conserved residues, R35T and K123A, and of the poorly conserved Arg²⁷, decreased IFN- β activities in most assays, suggesting an involvement of these residues in receptor interaction. While these results qualitatively mirror IFN- α mutational effects (32), the overall loss of activity from R35T and K123A is relatively small, indicating that some of the residues critical for IFN- α activity do not carry the same importance for IFN- β .

DISCUSSION

The aim of this study was to characterize the functional consequences of point mutations of IFN- β , which IFN- α mutational analyses and sequence comparisons had implicated as important for receptor interactions. We examined the biological activity of these mutant IFN- β s in assays that distinguish between IFN- α and - β subtypes, and in further assays that detect overall losses in activity (*i.e.* HL116 and WISH cells, which are equally sensitive to INF- α and - β subtypes). We primarily targeted solvent exposed residues of the AB loop, C helix and D helix, which are regions of the molecule shown to be important for IFN- α binding and function (32–34). We found that mutations in the NH₂-terminal AB loop and D helix had no effect on subtype-specific activities. Substitutions of residues highly conserved between IFN- α and - β s, R35T in the AB loop, and K123A in the D helix moderately reduced activity on human cells. By comparison with analogous mutations in IFN- α (32), these residues are relatively insensitive to mutation in

IFN- β , suggesting quantitatively different contributions of these residues to IFN- β -receptor interactions. We cannot exclude the possibility, that since much of the structure/function studies were performed on bacterially produced IFN- α s, those mutants may have been misfolded or less quantitatively assayed due to technological limitations (38–44). The eukaryotic cos cell expressed IFN- β mutants described in this study were soluble, highly glycosylated, and retained full activity over many months in conditioned medium. The importance of glycosylation for IFN- β stability and solubility have been recently described (53).

Heterologous systems have been used to define important domains of IFN- α necessary for cross-species reactivities, presumably by creating hybrid IFNs that interact better than parental forms with receptor components (42–44). We sought to extend these studies for human IFN- β by substituting AB loop residues, Arg²⁷, Tyr³⁰, and Arg³⁵, for equine IFN- β residues, since these residues are not well conserved between equine and human IFN- β s (Fig. 1). Neither substitutions of the AB loop nor C helix mutants showed increased activity on equine cells. This result implicates other regions of the molecule, possibly in the A helix or proximal D helix (homology comparisons shown in Fig. 1), as important determinants for equine receptor binding.

The introduction of charged residues at two positions in the C helix (N86E, N86K, Y92D, N86E,Y92D, and N86K,Y92D) resulted in IFN- β mutants with altered activities in several assays that distinguish between α and β subtypes. The tandem substitutions (N86E,Y92D and N86K,Y92D) had the most striking effect on subtype-specific activities, eliminating the IFN- β -induced association of phosphorylated ifnar1 and ifnar2 receptor chains in human Daudi Burkitt’s lymphoma cells, increasing antiviral activity on bovine MDBK cells, and lowering activity on tyk2-deficient human cells. While these changes represent a loss or decrease of specifically IFN- β characteristics and a shift toward the properties of IFN- α , it is not clear that they represent an acquisition of α -like properties. In particular, recent results show that the 11.1, tyk2-deficient cells from which the A27 strain was derived, express low levels of ifnar1 (45), and the C helix mutation may represent simply a reduced functionality in a specifically IFN- β -type interaction.

Considering that the two chains of the IFN receptor provide binding sites for different jak kinases, (12, 18–20) and STAT transcription factors, STAT1, STAT2 (46–48), and STAT3 (49), whose activities are induced by type I IFN binding, it is interesting to consider how alternative geometries of ligand-receptor complexes may achieve distinct signals through a common receptor.

STAT proteins bind to distinct receptor cytoplasmic domains and considerable overlap exists in their activation profiles in response to a wide spectrum of cytokines (46). Specificity of cytokine action may be achieved through finely tuned activation events mediated through specific receptor associations with Janus kinases, interdependent STAT binding and phosphorylation events, and differential assembly of homo- and heteromeric STAT complexes (21, 47, 48, 50), which distinguish promoter elements on the basis of their distinctive binding properties (21, 47). The potential for IFN- α s and - β s to differentially activate different STAT complexes, or to induce other signaling events (51, 52), could result in distinctive gene activation events. Further studies to delineate putative distinctive signaling events and differentially inducible genes will be necessary to test these possibilities.

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