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Human interferon gamma: significance of the C-terminal flexible domain for its biological activity

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Abstract

The significance of the C-terminal part of human interferon gamma (hIFNγ) for its biological activity was studied by 3′-end gene mutagenesis. A series of nine derivative genes obtained by systemic deletion of three codons was constructed and expressed in *Escherichia coli* LE392. It was shown that the yield of recombinant protein gradually decreased and the solubility gradually increased with truncation of the C terminus. To avoid artifacts related to the imperfect folding of the proteins during purification, the biological activity of the hIFNγ proteins was measured in clear cell lysates containing the soluble fractions only. The deletion of the C terminus had a two-step effect on both hIFNγ antiviral and antiproliferative activities. Whereas the removal of the last 3, 6, and 9 C-terminal amino acids led to a gradual increase (up to 10 times) in biological activity of hIFNγ, the deletion of more than 9 amino acids had an opposite effect. The truncation of the whole unstructured C-terminal domain resulted in a 10-fold decrease (but not in a complete loss) in biological activity of hIFNγ. The latter was sequestered upon deletion of 24 amino acids, 3 of which belonged to the α-helical domain F.

Keywords: Human interferon gamma; IFN-gamma; IFNγ; IFNγ C terminus; IFNγ biological activity; IFNγ structure–function relationship

Human interferon gamma (hIFNγ)[2] is endowed with multiple biological functions. In addition to its strong antiviral activity, it plays a key role in the modulation of immune response and is responsible for the defense against bacteria, intracellular parasites, and various xenobiotics [1].

Human IFNγ is a single-chain protein consisting of 143 amino acids and has a molecular mass of 17 kDa [2]. It is enriched in basic amino acids (18 lysines and 8 arginines) 8 of which are located in the C-terminal part of the molecule. The hIFNγ is organized in six α-helixes (marked from A to F) linked by short unstructured regions. Its C terminus extends from Pro^{122} to Gln^{143} and appears disordered or at least is highly flexible. The active form of hIFNγ is a homodimer in which two monomers are noncovalently bound in an antiparallel orientation [3].

The significance of the last 21 C-terminal amino acids for the activity of hIFNγ is quite disputable. Conclusions vary from "extremely important" to "totally dispensable." Although the spatial structure of hIFNγ was determined almost a decade ago [4], little is still known about the contribution of the unstructured C-terminal domain to the biological activity of this important cytokine.

Various methods, such as mutation analysis, partial enzymatic digestion, or use of specific monoclonal antibodies (MABs), have been employed to study the hIFNγ C terminus structure–function relationship. It

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[2] *Abbreviations used:* hIFNγ, human interferon gamma, MABs, monoclonal antibodies; ELISA, enzyme-linked immunosorbent assay; SSC, standard saline citrate; nt, nucleotide; NLS, nuclear localization sequence.

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has been shown that MABs specific to the last 9 [5], 15 [6], or 16 [7] C-terminal amino acids or even MABs recognizing the residues 121–130 [8] did not affect the hIFNγ antiviral activity. Based on these and other studies it was concluded that the unstructured C-terminal region of hIFNγ was dispensable for its biological activity. According to others, however, MABs specific to the amino acid residues 125–137 [9], 132–137 [10], or 130–138 [11] had a strong suppressive effect on both hIFNγ antiviral and hIFNγ antiproliferative activities.

Employing partial proteolysis with chymotrypsin, clostripain, or plasmin, Rinderknecht and Burton [12] obtained hIFNγ preparations truncated by 14 and 18 amino acids bearing 7% and less than 1%, respectively, of the antiviral activity of the intact protein. Using clostripain Leinikki et al. [13] prepared another form of hIFNγ shortened by 11 amino acids, which had 1000-fold lower receptor binding affinity, 50 times lower antiviral activity, and 10-fold lower ability to induce Fc receptors on human lymphoma cells. Based on these results it was concluded that the C terminus of hIFNγ was significant for the formation of receptor binding site. Other investigators have found that the proteolytic removal of 13 [14] and 15 [15] amino acids from the C terminus led to a 1000-fold decrease in the antiviral activity against the encephalomyocarditis virus and a substantial reduction in its affinity to the receptor. The results of Haelewyn et al. [16] showed that the hIFNγ receptor binding constant (Ka) of the proteolytically truncated by 14 C-terminal amino acids molecule was 1/24 of that of the intact protein. The removal of 1 more amino acid, i.e. 15, resulted in a complete loss of affinity to the receptor. It was also found in the same study that the deletion of 7 amino acids did not affect the hIFNγ antiviral activity. These observations indicated that Arg129 (the site of cleavage) was involved in the interaction of hIFNγ with its receptor. The latter conclusion contradicted the results of Honda et al. [17] showing that a hIFNγ truncated by 15 amino acids was fully active.

The results obtained by mutational analysis have also led to disputable conclusions. Sakaguchi et al. [18] constructed five derivative forms of hIFNγ devoid of 19 to 23 C-terminal amino acid residues and found that both their CD spectra and their antiviral activity were close to that of the intact hIFNγ. Similar results were obtained also by Slodowski et al. [19] where three other truncated forms of hIFNγ (lacking 14, 19, and 20 amino acids) were examined. They all possessed reduced but still detectable activity. Luk et al. [20] have also shown that the deletion of 21 C-terminal amino acids did not deprive hIFNγ of antiviral and antiproliferative activities. The deletion of 26, 32, and 37 C-terminal amino acids, however, resulted in a complete inactivation of hIFNγ. Based on these results it was concluded that the flexible C-terminal part of hIFNγ was not essential for its activity. According to others, truncated hIFNγ derivatives devoid of 19 or 23 C-terminal amino acids were deprived of biological activity [21]. This observation led to the conclusion that the intact C terminus was essential for maintaining the biological activity of hIFNγ. Such an assumption was supported by a series of studies showing that the C-terminal deletions decreased the ability of hIFNγ to inhibit both the growth of *Chlamidia trachomatis* and the replication of encephalomyocardiris and other viruses [22–24].

The extremely controversial data and conclusions concerning the role of the C-terminal domain in hIFNγ with regard to its biological activity stimulated us to undertake an independent study on this issue.

Materials and methods

Materials

Restriction endonucleases and other DNA-modifying enzymes were purchased from Gibco–BRL (USA) and protected phosphoramidites for oligonucleotide synthesis were bought from Dalton Chemicals (Canada). All other reagents for electrophoresis, chromatography, and purification of nucleic acids and proteins were products of Merck (Germany) and Sigma (USA). Oligonucleotides were synthesized on a Millipore Cyclon Plus DNA Synthesizer by the phosphoramidite method. Recombinant hIFNγ was obtained from *Escherichia coli* LE392 cells as previously described (Eur. Patent 0 446 582 B1, 04.01.1995). ELISA was performed with a hIFNγ sequence-specific monoclonal antibody obtained and characterized as already described [5].

Construction and expression of 3′-end truncated hIFNγ genes

To obtain 3′-end truncated hIFNγ genes, a gene coding for the full size of hIFNγ (143 amino acids) was amplified by a two-step PCR using a series of primers shown in Fig. 1. The 3′-end primers were designed to carry a stop codon TAA and a *Bam*HI restriction site. The 5′-end primer, common for all constructs, carried the initiation (ATG) codon and a *Hin*dIII cloning site. The series of truncated hIFNγ genes were then cloned in a pBR322-based expression vector, $pP_1SDIFN\gamma$, using the two *Hin*dIII and *Bam*HI cloning sites where *Hin*dIII preceded the initiation (ATG) codon and *Bam*HI (laying within the *tet* R gene of pBR322) followed the stop codon TAA. All constructs were sequenced on a DNA Cycle Sequencing System (Gibco–BRL). After verification of their nucleotide sequences the expression plasmids were transformed in *E. coli* LE392. SDS–polyacrylamide gel electrophoresis of lysates obtained from all transformed bacterial cells was performed as already described [5].

Forward (5'-end) primer:

5'-CCC*AAGCTT***ATG**CAGGACC-3';

Reversed (3'-end) primers:

1: 5'-CGC*GGATCC***TTA**ACGACGACCACGAAAC-3';

2: 5'-CGC*GGATCC***TTA**ACGAAACAGCATCTGCA-3';

3: 5'-CGC*GGATCC***TTA**CATCTGACTACGTTTAC-3';

4: 5'-CGC*GGATCC***TTA**ACGTTTACGCTTCCCT-3';

5: 5'-CGC*GGATCC***TTA**CTTCCCTGTTTTAGCCG-3';

6: 5'-CGC*GGATCC***TTA**TTTAGCCGCGGGCGA-3';

7: 5'-CGC*GGATCC***TTA**GGGCGACAGTTCTGC-3';

8: 5'-CGC*GGATCC***TTA**TTCAGCCATCACTTGGA-3';

9: 5'-CGC*GGATCC***TTA**CACTTGGATGAGTTCAT-3';

C: 5'-CGC*GGATCC***TTA**CTGGGATGCACGA – 3'

Fig. 1. Synthetic oligonucleotides used as primers for PCR amplification of the hIFNγ gene. The initiation codon (ATG in the forward primer) and the stop codon (TTA in the reverse primers) are boldfaced and the restriction sites (*Bam*HI and *Hin*dIII) are italicized. The numbers of the reversed primers correspond to the number of the truncated hIFNγ genes. C, reversed primer used for the construction of the hIFNγ full-size gene.

Protein yield determination

Samples of 20 ml Luria–Bertany medium supplemented with 50 μg/ml ampicillin were inoculated in a ratio of 1:50 with fresh overnight cultures of transformed *E. coli* LE392 cells and cultivated to a cell density of 0.7 or 2.0 OD_{595} at 37 °C. Bacteria were harvested by centrifugation and lysed by boiling in 7 M guanidine hydrochloride (GnHCl) for 2–5 min; protein concentration was determined by the Bradford [25] method. Cell lysates were diluted with phosphate-buffered saline (pH 7.2–7.4) to a final protein concentration of 27 μg/ml. Samples of 50 μl (in 11 repetitions) were coated on polyvinylchloride 96-well microplates (Costar Ltd., USA) by overnight incubation at 4 °C and the content of hIFNγ was determined by ELISA.

Determination of solubility of hIFNγ proteins in bacterial cytoplasm

Transformed cells were cultured as described above to a cell density of 0.7 or 2.0 OD_{595}. Bacterial cells (2.0 OD_{595}) were harvested, resuspended in 1 ml 0.14 M NaCl, 10 mM Tris–HCl, pH 8.0, and 0.1 mM phenylmethylsulfonyl fluoride, and disrupted by sonication. After centrifugation (15 min, 14,000*g*) the supernatants (clear lysates) were collected and the pellets were dissolved in 1 ml 7 M GnHCl. The content of hIFNγ in both fractions (soluble and insoluble) was determined by ELISA.

Determination of hIFNγ mRNA

Transformed bacteria were cultured to a cell density of 0.7 OD_{595}. Total RNA was isolated from 2.0 OD_{595} cells (using Trizol reagent, Gibco–BRL) and dissolved in 25 μl of RNase-free water. Samples of 5 μg RNA from each construct (in three repetitions) were spotted on a Hybond N^+ membrane (Amersham) and cross-linked by UV. The filters were prehybridized in hybridization buffer (0.1 M Tris–HCl, pH 8, 0.1% SDS, and 6× SSC) supplemented with 100 μg/ml sonicated and denatured salmon sperm DNA for 2 h at the temperature of hybridization. The latter was determined by the formula $T_h = T_m - 5\,°C$, where T_m is the melting temperature of the hybrid with the corresponding oligonucleotide. A 19-nt ^{32}P-labeled oligonucleotide specific to the hIFNγ gene was then added and the hybridization was carried out for 2 h at the same temperature. The filters were washed three times for 10 min with the hybridization buffer at a temperature 10 °C lower than the T_h and scanned on a β-SCAN radioanalytic instrument (Instant-Imager, Packard Instrument Co). The ^{32}P-labeled probe was then removed by four 10-min incubations at 65 °C in 50% formamide, 1% SDS, 0.1× SSC, and 40 mM Tris–HCl, pH 7.8, at 65 °C and one incubation in the same solution free of formamide. For normalization, the same filters were rehybridized with a 17-nt oligonucleotide specific for the *E. coli* 16S rRNA.

Determination of hIFNγ biological activity

Clear lysates were obtained from *E. coli* LE392 cells expressing hIFNγ genes as already described, sterilized by filtration and subjected to serial dilutions appropriate for the performance of biological activity assays. Antiviral activity of hIFNγ (in clear lysates) was determined by measuring the protective effect on WISH cells against the cytopathic action of the vesicular stomatitis virus as described by Forti et al. [26] with slight modifications [5]. Antiproliferative activity was determined by a modified kynurenine bioassay as described recently [27]. To determine the specific activity of the investigated hIFNγ the protein content in the lysates was determined by the Bradford [25] method and the concentration of hIFNγ was measured by ELISA using purified recombinant hIFNγ (99.5% purity and specific antiviral activity 5×10^7 IU/mg) as a standard.

Results and discussion

The literature survey shows that there is great inconsistency in conclusions with regard to the significance

of the unstructured C-terminal domain for the biological activity of hIFNγ. Whereas the controversy of the results obtained when different methodology is employed might be explained by differences in the specificity and resolution of the methods used, it is not easy to explain the discrepancy in the results in the cases when identical methodology (e.g., recombinant DNA approach) has been applied. Taking into consideration that the specific biological activity of a recombinant hIFNγ preparation depends very much on its correct folding, we are tempted to explain the discrepancy of the literature results by the different specific activities of the hIFNγ preparations used. Bearing in mind the possible sources of experimental artifacts, we decided to undertake a new study on the role of the hIFNγ C terminus based on a systematic 3′-end deletion (by three codons) of the hIFNγ gene (coding originally for 143 amino acids). To this end a series of nine truncated hIFNγ gene constructs (designated hIFNγΔ1 to hIFNγΔ9) were created by PCR using appropriate primers (see Fig. 1). They were cloned in a pBR322-based constitutive expression vector carrying a synthetic constitutive promoter P1 and a consensus SD ribosome-binding site. As seen in Fig. 2, the construct hIFNγΔ7 was designed to code for a protein completely devoid of unstructured C terminus (21 amino acids missing) and the constructs hIFNγΔ8 and hIFNγΔ9 were made to code for proteins lacking 24 and 27 amino acids, respectively; 3 and 6 of the amino acids in the latter two constructs belonged to the last (F) α-helical domain. The efficiency of expression of the wild-type and all truncated hIFNγ proteins in *E. coli* LE392 cells is shown in Fig. 3.

The yield of the recombinant hIFNγ proteins was measured by ELISA. As seen in Table 1, the protein yield obtained from the truncated constructs was lower than that of the wild-type (full-size) hIFNγ expressed

IFNγC

Gln[1] ... ... Val Met Ala Glu Leu[120] Ser *Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg Ser Gln Met Leu Phe Arg Gly Arg Arg[140] Ala Ser Gln*

IFNγΔ1

Gln[1] ... ... Val Met Ala Glu Leu[120] Ser *Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg Ser Gln Met Leu Phe Arg Gly Arg Arg[140]*

IFNγΔ2

Gln[1] ... ... Val Met Ala Glu Leu[120] Ser *Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg Ser Gln Met Leu Phe Arg*

IFNγΔ3

Gln[1] ... ... Val Met Ala Glu Leu[120] Ser *Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg Ser Gln Met*

IFNγΔ4

Gln[1] ... ... Val Met Ala Glu Leu[120] Ser *Pro Ala Ala Lys Thr Gly Lys Arg Lys[130] Arg*

IFNγΔ5

Gln[1] ... ... Val Met Ala Glu Leu[120] Ser *Pro Ala Ala Lys Thr Gly Lys*

IFNγΔ6

Gln[1] ... ... Val Met Ala Glu Leu[120] Ser *Pro Ala Ala Lys*

IFNγΔ7

Gln[1] ... ... Val Met Ala Glu Leu[120] Ser *Pro*

IFNγΔ8

Gln[1] ... ... Val Met Ala Glu

IFNγΔ9

Gln[1] ... ... Val

Fig. 2. C-terminal amino acid sequence of hIFNγ derivative proteins. The amino acids belonging to the flexible C terminus are presented in italic. Numbering begins with glutamine (the first N-terminal amino acid in the mature protein).

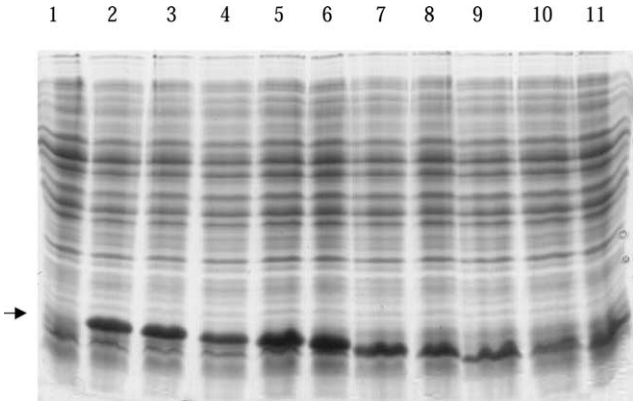

Fig. 3. SDS–polyacrylamide gel electrophoresis of crude lysates of *E. coli* LE392 cells expressing hIFNγ derivative genes. Lane 1, *E. coli* LE392 host (nontransformed) cells; lanes 2–11, bacterial cells expressing hIFNγC, hIFNγΔ1, hIFNγΔ2, hIFNγΔ3, hIFNγΔ4, hIFNγΔ5, hIFNγΔ6, hIFNγΔ7, hIFNγΔ8, and hIFNγΔ9 genes, respectively. Arrow indicates the position of the hIFNγ.

from the same vector. These data supported the observations of Slodowski et al. [19] showing that the yield of truncated hIFNγ proteins in general was lower than that of the control. In our case the yield of truncated proteins varied from 60 to 57% for the constructs with 3 to 15 C-terminal amino acids missing and was lower than 45% for the shorter constructs. Unfortunately we were unable to determine quantitatively the protein yield obtained from the shortest gene constructs (hIFNγΔ8 and hIFNγΔ9) because of the inability of the MAB to recognize hIFNγ proteins shorter than 122 amino acids but the overexpression of these proteins was well visible on SDS–polyacrilamides gels (Fig. 3).

Since the observed variations in the protein yields could be due to the different yields of mRNA, the relative yield of hIFNγ mRNA was also measured. The results presented in Table 1 show that the deviation in the yield of hIFNγ mRNA from the different constructs was even greater than that of the protein yield. The highest yield of mRNA was obtained from the construct hIFNγΔ1 (157.1% compared to the control). High content of mRNA was also observed with the constructs hIFNγΔ7 (125.2%) and hIFNγΔ2 (104.7%), whereas the lowest yield was registered with the construct hIFNγΔ4 (55.8%).

The variations in the yield of mRNA could be explained by variations in either transcription efficiency or stability of mRNA. Our attempts to find out differences in the stability (half-life) of mRNA, after blocking the transcription with rifampicin and nalidixic acid, failed because of the extremely short half-life (about 60 s) of all hIFNγ mRNAs examined and the low resolution of the method applied (±10–15 s).

The normalized yield of protein (protein yield related to the yield of mRNA) showed that the most efficient translation was with the mRNA obtained from the construct hIFNγΔ4 followed by that of the control (hIFNγ full-size gene) and the construct hIFNγΔ5 (Table 1). The least efficient was the translation of the mRNAs corresponding to the constructs hIFNγΔ7 and hIFNγΔ1.

Prior to measuring biological activity, we determined the solubility of the derivative hIFNγ proteins in the *E. coli* cytoplasm. It is well known that the recombinant full-size hIFNγ tends to aggregate into insoluble inclusion bodies. To estimate the influence of the unstructured C-terminal domain on the aggregation ability of hIFNγ, the recombinant proteins were separated into two fractions (soluble and insoluble) after mild lysis of the *E. coli* LE392 cells. The latter were harvested at two different stages of the growth phase ($A_{595} = 0.7$ and 2.0) and the content of recombinant protein was determined by ELISA. Table 2 shows that the systematic shortening of the C-terminal domain by zero to 21 amino acids led to a gradual increase in solubility of the corresponding protein from 46.6 to 66.7% for the exponential phase ($A_{595} = 0.7$) and from 24.0 to 41.5% for the stationary phase ($A_{595} = 2.0$). Although such increase was observed by others [19,28] this phenomenon was not so simple to explain.

Table 1
Relative yield of truncated hIFNγ proteins and mRNA

Construct	Relative yield of hIFNγ (%)[a]	Relative yield of hIFNγ mRNA (%)[b]	Normalized yield of hIFNγ (%)[c]
IFNγC (control)	100	100	100
IFNγΔ1	60.1 ± 0.35	157.1 ± 0.41	38.3 ± 0.24
IFNγΔ2	60.3 ± 0.28	104.7 ± 0.38	57.6 ± 0.34
IFNγΔ3	57.1 ± 0.4	85.4 ± 0.35	66.9 ± 0.54
IFNγΔ4	59.2 ± 0.32	55.8 ± 0.27	106.1 ± 0.77
IFNγΔ5	57.3 ± 0.27	69.5 ± 0.19	82.4 ± 0.45
IFNγΔ6	45.2 ± 0.19	83.1 ± 0.31	54.4 ± 0.31
IFNγΔ7	37.8 ± 0.41	125.2 ± 0.20	30.2 ± 0.33
IFNγΔ8	n.d.	77.5 ± 0.29	n.d.
IFNγΔ9	n.d.	73.0 ± 0.44	n.d.

The yield of protein ([a]) determined by ELISA or hIFNγ mRNA ([b]) determined by hybridization is related to that obtained with the construct hIFNγC (full size gene) taken as 100%. [c]The yield of protein is related to the yield of hIFNγ mRNA; n.d., not determined.

Table 2
Solubility of hIFNγ derivative proteins in *E. coli* LE392 cytoplasm

Construct	Exponential growth (0.7 OD_{595})		Stationary phase (2.0 OD_{595})	
	Soluble protein (%)[a]	Insoluble protein (inclusion bodies) (%)[b]	Soluble protein (%)[a]	Insoluble protein (inclusion bodies) (%)[b]
IFNγC	46.6 ± 0.12	53.4 ± 0.31	24.0 ± 0.09	76.0 ± 0.1
IFNγΔ1	47.3 ± 0.19	52.3 ± 0.2	27.0 ± 0.12	73.0 ± 0.15
IFNγΔ2	44.2 ± 0.11	55.8 ± 0.18	33.5 ± 0.15	66.5 ± 0.16
IFNγΔ3	52.8 ± 0.17	47.2 ± 0.11	39.8 ± 0.11	60.2 ± 0.21
IFNγΔ4	53.7 ± 0.12	46.3 ± 0.21	41.2 ± 0.23	58.8 ± 0.24
IFNγΔ5	62.3 ± 0.2	37.7 ± 0.15	41.6 ± 0.3	58.4 ± 0.09
IFNγΔ6	56.5 ± 0.22	43.6 ± 0.17	41.6 ± 0.17	58.4 ± 0.14
IFNγΔ7	66.7 ± 0.3	33.3 ± 0.2	41.5 ± 0.19	58.5 ± 0.16
IFNγΔ8	n.d.	n.d.	n.d.	n.d.
IFNγΔ9	n.d.	n.d.	n.d.	n.d.

The portion of soluble ([a]) and insoluble ([b]) protein is presented as a part (in %) of the total protein determined by ELISA for each hIFNγ construct; n.d., not determined.

More than one half of the amino acids belonging to the hIFNγ C-terminal domain are hydrophilic (three Lys, five Arg, two Ser, and one Thr) and therefore it is logical to expect that their deletion would result in an increase in the overall hydrophobicity of the molecule. On the other hand, the latter should increase the tendency of hIFNγ to aggregate. To explain the extraordinary high tendency of hIFNγ to aggregate we have recently studied the electrostatic interactions in hIFNγ at two different physical states: free dimer and dimer associated with the hIFNγ receptor [29]. Our calculations based on the three-dimensional structure showed that, in addition to the highly hydrophilic pattern of hIFNγ (due to the presence of 54 titratable and 20 nontitratable hydrophilic groups), most of its titratable groups were buried inside the molecule. They are highly dehydrated and therefore remain neutral (unionized), which is the reason for the overall domination of hydrophobic interactions between the free dimers in solution leading to a rapid aggregation.

The C terminus of hIFNγ is unstructured and accessible to the solvent. Consequently, its Arg and Lys residues are protonated and could neutralize partly the negative charges of some titratable groups exposed to the surface of the molecule. Such interactions should result in an additional increase in the hydrophobicity of hIFNγ. When the C-terminal amino acids are deleted, their neutralizing effect is sequestered, leading to an increase in the total negative charge and an impediment of protein aggregation.

As mentioned above, to avoid artifacts related with the incorrect folding (accompanying protein purification), we have measured the biological activity of the C-terminally truncated hIFNγ proteins in clear sterile cell lysates. Thus the insoluble fraction (inclusion bodies) was completely ignored and the specific biological (both antiviral and antiproliferative) activity was determined on the basis of the protein concentration in the cytosol only. The results presented in Table 3 show that this approach was quite reasonable. As seen from the table, the specific activity of the full-size hIFNγ in the soluble fraction was more than one order of magnitude higher than that of the same protein (used as a reference) after purification. Although the hIFNγ folding problem is beyond the scope of this study, it should be mentioned that it is still unresolved and remains one of the main obstacles in the way of both mass production and wide clinical application of the recombinant hIFNγ.

Table 3
Specific antiviral and antiproliferative activity of hIFNγ derivative proteins

Construct	Antiviral bioassay (IU/mg)[a]	Kynurenine bioassay (IU/mg)[b]
IFNγC	$(2.2 \pm 1.5) \times 10^8$	$(2.8 \pm 0.21) \times 10^8$
IFNγΔ1	$(2.5 \pm 1.3) \times 10^8$	$(3.9 \pm 0.25) \times 10^8$
IFNγΔ2	$(2.7 \pm 1.7) \times 10^8$	$(6.0 \pm 0.39) \times 10^8$
IFNγΔ3	$(1.16 \pm 2.1) \times 10^9$	$(3.2 \pm 0.11) \times 10^9$
IFNγΔ4	$(5.0 \pm 1.1) \times 10^7$	$(7.4 \pm 0.4) \times 10^7$
IFNγΔ5	$(8.2 \pm 1.3) \times 10^7$	$(7.9 \pm 0.37) \times 10^7$
IFNγΔ6	$(2.9 \pm 1.5) \times 10^7$	$(2.9 \pm 0.42) \times 10^7$
IFNγΔ7	$(6.1 \pm 1.6) \times 10^7$	$(2.1 \pm 0.33) \times 10^7$
IFNγΔ8	0	0
IFNγΔ9	0	0

In both antiviral ([a]) and antiproliferative ([b]) bioassays a purified recombinant hIFNγ (obtained from inclusion bodies isolated from *E. coli* LE392 cells) with a specific antiviral activity of 5×10^7 IU/mg was used as a reference; n.d., not determined.

As seen in Table 3, the C-terminal deletion of amino acids had a two-step effect on the biological activity of hIFNγ. At the beginning it led to a gradual increase in both antiviral and antiproliferative activities, approaching maximal values (1.16×10^9 and 3.2×10^9 IU/mg, respectively) upon deletion of 9 amino acids (as in the construct hIFNγΔ3). Similar observations have also been made by others for hIFNγ proteins lacking 9–11 C-terminal amino acids [19,23,24,30,31]. We assume that the elimination of nine amino acids (3 of which are Arg) makes some basic amino acids from the rest of the

molecule more accessible for interaction with the hIFNγ receptor. This would lead to an increase of biological activity of the truncated derivative.

Further deletions, however, had an opposite effect, resulting in a 10-fold decrease in specific activity compared to that of the control. The lowest antiproliferative activity (2.1×10^7 IU/mg) was registered upon deletion of the whole unstructured C-terminal domain (hIFNγΔ7). It is important to emphasize, however, that although the truncation of 12 to 21 C-terminal amino acids resulted in a substantial decrease in both antiviral and antiproliferative activities, the corresponding hIFNγ proteins had their biological activity still preserved. The next deletions affecting the α-helix F (as in the constructs hIFNγΔ8 and hIFNγΔ9), however, led to a complete loss of hIFNγ activity.

Combining the results presented above with the literature data, we can conclude that the deletion of 12 or more amino acids from the C terminus of hIFNγ results in a substantial loss of biological activity. This means that the sequence 122–134 (including the basic amino acids Lys128, Arg129, Lys130, Arg131), which is considered dispensable for the formation of functional hIFNγ dimer, is significant for the overall biological functions of the protein. This conclusion is supported by studies using MABs for investigation of antiviral and antiproliferative activities of hIFNγ [9,10,32] and by other studies using heparin sulfate for investigation of its interaction with the receptor [24,33,34]. Employing synthetic peptides, it has been also shown that the C-terminal sequence 95–134 interacts with the cytoplasmic domain of hIFNγ receptor in a non-species specific manner [32,35,36]. Similar data directed at the extracellular part of hIFNγ receptor were obtained by Walter and co-workers [4]. Investigating the crystal structure of the complex hIFNγ/hIFNγ soluble receptor, these investigators have suggested that the amino acids involved in this interaction are located between the residues 1–42 and 108–124. The first segment contains helix A, the AB loop, and helix B and the second segment contains helix F and a part of the C terminus. However, these authors mentioned that, although their model was capable of explaining the general requirement of hIFNγ for having a basic charge at its C terminus (necessary for high-affinity binding), it was unable to identify any specific interaction between the C terminus and the hIFNγ receptor.

A recent finding showing that a deletion mutant of hIFNγ lacking 23 C-terminal amino acids is biologically inactive was explained by the absence of the putative C-terminal nuclear localization sequence (NLS) [37]. This sequence located between the amino acid residues 95 and 134 was found to be involved in the interaction of hIFNγ with the cytoplasmic domain of the α chain of its receptor [38] and with the transcription activator STAT1 [39]. In an early study the basic sequence 128–131 was described as responsible for the nuclear accumulation of hIFNγ [40].

These findings, however, cannot explain the data reported here with regard to residual (10 times lower) activity of the constructs devoid of 12 to 21 C-terminal amino acids in which the putative NLS is either incomplete or completely missing. Similar results were also reported by Sakaguchi et al. [18], Luk et al. [20], Michalski et al. [41], and Slodowsky et al. [19]. The latter investigators observed a decrease in biological activity of the proteins truncated by more than 14 C-terminal amino acids. These deletions resulted in a 100-fold decrease in biological activity but not in a complete inactivation of hIFNγ. Probably the observed activity of similar proteins lower than those presented in our study was related to the specificity of the procedures applied for purification and folding of the hIFNγ derivatives with different C-terminal truncations. Moreover, the here reported drastic leap in biological activity of hIFNγ proteins truncated by 21 ($6.1–2.1 \times 10^7$ IU/mg) and 24 (no biological activity) amino acids could not be explained by the lack of NLS since both proteins were deprived of this sequence. The complete loss of activity in the latter case might be due to either lack of some important component for binding of hIFNγ to the receptor or formation of altered tertiary structure related to the truncated α-helix F. We assume that such could also be the reason for the absence of biological activity in the construct of Subramanian et al. [37], lacking 23 C-terminal amino acids, despite its reported capability for binding to the extracellular part of the hIFNγ receptor.

An alternative upstream NLS sequence, located between the amino acid residues 84 and 94 in hIFNγ and resembling the NLSs of a number of nuclear localization proteins (such as the SV40 large tumor antigen, polyoma virus, and the steroid hormone receptors), was described a long time ago by Zu and Jay [42]. Larkin and co-workers [43] found that this sequence was less efficient than the downstream (C-terminal) NLS, which was consistent with the observed loss of more than 90% of biological activity in the protein lacking the downstream NLS. It is worth mentioning, however, that this investigation had been carried out using synthetic peptides as competitors of the two NLSs and not by mutagenesis as in the present study.

Recently an Arg/Lys-rich motif was found in the STAT1 molecule lying within its DNA-binding domain. By a series of point mutations it was shown that two Arg/Lys-rich elements, one in each STAT1 monomer, but not in the C terminus of hIFNγ, were required for the hIFNγ-induced nuclear import of the STAT1 dimer [44]. This finding means that, despite the great amount of results obtained up to now, our knowledge on the intracellular cascade of events induced by hIFNγ is still incomplete. Based on the results reported here, we postulate that, although the potential downstream NLS

in hIFNγ seems to be important for its nuclear localization, its absence is compensated by some other factor (probably the upstream NLS), which protects this important cytokine from complete loss of activity. The hIFNγ is deprived of biological activity only in the case when its α-helixes are affected.

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