Interaction of truncated human interferon γ variants with the interferon γ receptor: crucial importance of Arg-129

Joost HAELEWYN*, Lieve MICHIELS*, Peter VERHAERT†, Marc F. HOYLAERTS‡, Raphaël WITTERS* and Marc DE LEY*§

*Laboratory for Biochemistry, Katholieke Universiteit Leuven (KULeuven), Celestijnenlaan 200 G, B-3001 Leuven-Heverlee, Belgium, †Laboratory for Developmental Physiology and Molecular Biology, Zoological Institute, KULeuven, Naamsestraat 59, B-3000 Leuven, Belgium, and ‡Center for Molecular and Vascular Biology, KULeuven, Herestraat 49, B-3001 Leuven-Heverlee, Belgium

Recombinant human interferon γ (IFN- γ), produced in *Escherichia coli*, was selectively truncated at its C-terminus with chymotrypsin, clostripain or plasmin. The C-terminal amino acid residues of the three truncated IFN- γ variants were identified as Phe¹³⁶, Arg¹²⁹ and Lys¹²⁸, indicating the removal of 7, 14 and 15 amino acid residues from the full-length molecule. The absence of seven C-terminal residues did not influence the binding of IFN- γ to its receptor. In contrast, the truncation of 14 residues

resulted in a decrease in the K_a value to 1/24, as determined by surface plasmon resonance analysis. The removal of one additional amino acid residue from the C-terminal region of IFN- γ led to a marked loss of receptor-binding capacity and biological activity. These observations demonstrate that Arg¹²⁹ is an essential part of a functionally important C-terminal IFN- γ sequence that is involved in receptor interaction.

INTRODUCTION

Human interferon γ (IFN- γ), a cytokine that is produced by activated T-cells and natural killer cells, exerts its pleiotropic biological effects by binding to a specific receptor protein at the target cell surface [1]. One IFN- γ dimer, consisting of two identical polypeptide chains of 143 amino acid residues, is capable of binding two receptor proteins [2]. Some of the residues that are involved in the interaction have been identified by X-ray crystallography of the IFN- γ -receptor complex [3]; particularly, it was found that the residues at positions 23–26, which are part of a loop between two α -helices in the IFN- γ molecule, interact extensively with the receptor. However, the X-ray data did not elucidate the contribution of the C-terminal portion of the IFN- γ molecule to receptor binding. The C-terminal part of IFN- γ is a highly positively charged, solvent-exposed tail, lacking a rigid conformation [4], and is highly susceptible to proteolytic processing [5,6]. By altering and truncating parts of the C-terminal region a functionally important sequence has been located: the replacement of the four basic residues at positions 128–131 by four Ser residues resulted in a decrease in the antiviral activity of IFN- γ to 1/10–1/30 [7]. Two nonsense mutants, both lacking 19 residues, displayed only 0.05–1.5% of the biological activity of wild-type IFN- γ [7,8]. In contrast, it was observed that the truncation of 10 [9], 11 [8] or even 12 [10,11] residues from the C-terminal region of IFN- γ influenced its activity only slightly. In this study three truncated IFN- γ forms have been prepared by the use of proteases with high substrate specificity. The resulting preparations were unambiguously identified and characterized by matrix-assisted laser desorption/ionization MS (MALDI-MS) and measurements of surface plasmon resonance. Our interaction studies with these truncated IFN- γ forms have yielded a more detailed picture of the C-terminal amino acid residues that are involved in receptor binding.

EXPERIMENTAL

Materials

Recombinant human IFN- γ was produced in *Escherichia coli* and purified to homogeneity as described previously [12]. Recombinant soluble IFN- γ receptor, consisting of the extracellular region of the native protein (ECR), was expressed in J558L cells as a fusion protein with a tag-peptide and purified from the cell supernatant by means of affinity chromatography on the immobilized monoclonal antibody MYC-9E10.2 (directed against the tag-peptide); the tag-peptide does not interfere with the binding of IFN- γ to its receptor. Chymotrypsin, plasmin, aprotinin and carboxypeptidase P (CPP) were purchased from Boehringer (Mannheim, Germany); clostripain was from Sigma (St. Louis, MO, U.S.A.).

Preparation and purification of C-terminally truncated IFN- γ

Limited chymotryptic digestion of full-length IFN- γ was achieved by incubation of the mixture at an enzyme-to-IFN- γ ratio of 1:1000 (w/w) in PBS for 15 min at room temperature. The digestion was stopped by the addition of aprotinin at an inhibitor-to-enzyme ratio of 5:1 (w/w). Treatment of IFN- γ with clostripain has been described elsewhere [13]. Digestion with plasmin was performed at an enzyme-to-IFN- γ ratio of 1:10 (w/w) in PBS for 4 h at 37 °C, as previously reported [9]. The resulting IFN- γ fragments were purified by gel filtration on a Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden), run at 0.5 ml/min with PBS, except for the chymotryptic digestion product, which was purified in the presence of an additional 0.1 M NaCl.

MALDI-MS

Purified IFN- γ fragments were prepared for MALDI-MS by

Abbreviations used: CPP, carboxypeptidase P; ECR, the soluble extracellular part of the IFN- γ receptor; IFN- γ , interferon γ ; MALDI, matrix-assisted laser desorption/ionization.

§ To whom correspondence should be addressed.

reverse-phase HPLC on a Zorbax Protein Plus column (25 cm $\times$ 4.6 mm; Du Pont, Wilmington, DE, U.S.A.) and eluted with a linear gradient of 0–64 % (v/v) acetonitrile in 0.1 % (v/v) trifluoroacetic acid at a flow rate of 0.8 ml/min over 60 min. The MALDI-MS spectra were obtained with a VG TofSpec (Micromass, Manchester, U.K.). Spectra were externally calibrated with cytochrome *c* and horse heart myoglobin. Ions, desorbed by pulses from a N_2 laser (337 nm), were accelerated at 24 kV (positive ion mode). After a linear flight path of 0.65 m, ions were detected by a hybrid micro-channel plate/electron-multiplier detector.

C-terminal amino acid determination

The C-termini of purified truncated IFN- γ forms were determined via enzymic digestion by CPP and subsequent amino acid analysis [14]. Each analysis was started after the addition of 20 nmol of norleucine to 0.5 mg of the protein sample [1 mg/ml in 50 mM sodium acetate buffer (pH 4.0)/0.02 % (v/v) Triton X-100]. A 125 μ l aliquot, serving as a negative control, was taken, mixed with 250 μ l of 20 % (v/v) TFA and centrifuged for 10 min. The supernatant was transferred into another vial and dried under vacuum. Then CPP was added to the rest of the protein sample at an enzyme-to-protein ratio of 1:150 (mol/mol) and incubated at room temperature. Three more samples were taken at 2, 5 and 10 min respectively and further treated as described. The released amino acids were analysed on an LKB 4150 Alpha Automatic Amino Acid Analyser (Bromma, Sweden) with ninhydrin-mediated detection.

Competition ELISA

This technique was used to compare the C-terminally truncated IFN- γ products with respect to their receptor-binding affinity, with the following configuration: plates were coated overnight with intact IFN- γ (3 μ g/ml) in 50 mM $NaHCO_3$ buffer, pH 9.7, at 4 °C, and saturated with 1 % (w/v) casein. Intact IFN- γ and its derived variants were applied in serial 1:2 dilutions in the presence of 1 μ g/ml ECR. After 1 h of incubation at room temperature, detection of ECR was performed with a rabbit anti-ECR antiserum, followed by an alkaline phosphatase-conjugated sheep anti-rabbit antiserum (Boehringer), with *p*-nitrophenyl phosphate as the substrate. Absorbance was measured at 405 nm.

Antiviral assay

The IFN- γ variants were sequentially diluted in a 96-well plate in minimum essential medium containing 2 % (v/v) newborn calf serum; human lung carcinoma A549 cells [15] were added (50 000 cells per well). After 16 h of incubation the medium was replaced and EMC virus was added (2.4×10^4 plaque-forming units per well). When the cytopathic effect of the virus in a control well (no IFN- γ) had exceeded 90 %, the medium was removed; the cells were fixed with formaldehyde and stained with Naphthol Blue Black. After repeated washes with tap water the dye was extracted from the cells with 50 mM NaOH, after which absorbance was measured at 620 nm.

Measurement of surface plasmon resonance

Real-time kinetic analysis of the IFN- γ -receptor interaction was performed with a Pharmacia Biosensor BIAcore® instrument. Recombinant ECR (12 μ g/ml in 10 mM sodium acetate buffer, pH 4.0) was immobilized on the activated surface of a sensor chip as described previously [16]. All binding experiments were performed in Hepes-buffered saline [10 mM Hepes (pH 7.4)/

150 mM NaCl/3.4 mM EDTA/0.05 % (v/v) Tween-20] at a constant flow rate of 10 μ l/min. Intact and truncated IFN- γ were injected on the ECR-immobilized sensor chip surface at various concentrations (250, 125 and 62 nM). At the end of each run cycle the surface was regenerated with 10 mM HCl. The association and dissociation rate constants were determined with the BIA evaluation software package.

RESULTS AND DISCUSSION

Selective proteolysis at the IFN- γ C-terminal tail

Purified *E. coli*-derived IFN- γ was subjected to limited proteolysis at its C-terminus, with chymotrypsin, clostripain or plasmin. The mobilities of the intact molecule and its purified derivatives under SDS/PAGE are shown in Figure 1. Intact IFN- γ appears as an 18 kDa band, corresponding to its monomeric state on treatment with SDS. Truncation of IFN- γ at its C-terminus led to the appearance of a second band of higher M_r , corresponding to dimeric IFN- γ , which has been observed before [13,17,18]. For each variant one single monomer band was observed, suggesting the formation of a stable fragment of slightly lower M_r than the intact molecule.

To verify the exact cleavage site(s) of the truncated IFN- γ variants, C-terminal amino acid determination was performed, as well as molecular mass analyses by means of MALDI-MS. The spectra are shown in Figure 2; the observed M_r values refer to single polypeptides. Analysis of the full-length polypeptide showed a narrow peak with a mass/charge (m/z) ratio for the singly protonated molecular ion of 16926 ± 50 (Figure 2a), which is in good agreement with the calculated M_r (16908). Analysis of chymotrypsin-treated IFN- γ revealed a somewhat broader m/z peak with a maximum value at 16083 ± 100 (Figure 2b), which corresponds to an IFN- γ fragment terminating at Phe¹³⁶ (calculated M_r 16098); this is in agreement with the substrate specificity of the protease employed. Additional cleavage of the Tyr⁴-Val⁵ bond at the N-terminus of the molecule might explain the presence of a second signal at 15607 ± 100 . It has previously been demonstrated that selective N-terminal proteolysis with *Staphylococcus aureus* V8 protease (cleavage at Glu⁷-Ala⁸ and Glu⁹-Ala¹⁰) causes alterations in the secondary structure of IFN- γ and greatly decreases its antiviral activity [18,19]. However, the experiments described below will show that the partial omission of the Met⁰-Tyr⁴ sequence does not affect the activity of IFN- γ . A C-terminal amino acid determination of chymotrypsin-treated IFN- γ identified phenylalanine as the C-terminal residue (results

Figure 1 SDS/PAGE [under reducing conditions on a 17.5 % (w/v) gel] after limited proteolysis of full-length IFN- γ with different enzymes

Lane 1, intact IFN- γ ; lane 2, IFN- γ after treatment with chymotrypsin; lane 3, clostripain; lane 4, plasmin; lane M, M_r markers (values indicated at the left).

Figure 2 Molecular mass determination of intact IFN- γ and its proteolytic digestion products by MALDI-MS

(a) Intact IFN- γ ; (b) IFN- γ after limited chymotryptic digestion; (c) IFN- γ after treatment with clostripain; (d) IFN- γ after digestion with plasmin. IFN- γ polypeptides are $[M + H]^+$ ions; m/z values are indicated.

Figure 3 Determination of the C-terminal amino acid of IFN- γ after treatment with clostripain (upper panel) and plasmin (lower panel)

The amounts of released amino acids (nmol) are plotted as a function of time (min): $\square$, Arg; $\circ$, Lys; $\triangle$, Gly; $\diamond$, Thr.

not shown), confirming the MALDI-MS analysis. The fragment is designated below as IFN- $\gamma\Delta 7$.

MALDI-MS analysis of IFN- γ digested with clostripain showed a signal with an m/z of 15249 ± 50 (Figure 2c). This value could be assigned to an IFN- γ fragment produced by cleavage at the Arg¹²⁹-Lys¹³⁰ bond (calculated M_r 15205) but not to a cleavage at Lys¹²⁸-Arg¹²⁹ (calculated M_r 15050), as had been proposed previously [13]. An analogous discrepancy was observed in the case of IFN- γ treated with plasmin (Figure 2d): the observed m/z of 15093 ± 50 did not correspond to the formerly proposed Met⁰-Lys¹²⁵ polypeptide [9]; rather it matches a polypeptide terminating at Lys¹²⁸ (calculated M_r 15050).

To confirm experimentally the exact cleavage sites recognized by both these enzymes, C-terminal amino acid determinations were performed by means of exopeptidase digestion with CPP and analysis of the released amino acids. Figure 3 (upper panel) (IFN- γ treated with clostripain) shows that Arg is the first residue to be released by CPP, followed by a Lys residue. This result unambiguously confirms the MALDI-MS analysis: Arg¹²⁹ (and not Lys¹²⁸) is the C-terminal residue of the clostripain-treated IFN- γ . The fragment is designated below as IFN- $\gamma\Delta 14$. The small amounts of Gly (and consequently Thr) detected can be explained by the low hydrolysis rate of this residue by CPP. No other amino acids were detected. Figure 3 (lower panel) shows the C-terminal amino acid determination of IFN- γ digested with plasmin. The C-terminal amino acid of this fragment is mainly Lys, although a small amount of Arg was also

Figure 4 Interaction of intact IFN- γ ($\square$) and its C-terminally truncated derivatives IFN- γ Δ 7 ($\circ$), IFN- γ Δ 14 ($\triangle$) and IFN- γ Δ 15 ($\diamond$) with the IFN- γ receptor

Upper panel: binding to soluble recombinant ECR, measured by a competition ELISA. A positive (no IFN- γ ; broken line) and a negative control (no ECR; dotted line) are also shown. Lower panel: antiviral activity; 50% inhibition of the cytopathic effect was observed at concentrations of 0.021 nM (intact dimeric IFN- γ), 0.013 nM (IFN- γ Δ 7) and 0.89 nM (IFN- γ Δ 14). No 50% inhibition was reached for IFN- γ Δ 15 (the maximum concentration applied was 12 nM). A cell control (no virus, broken line) and a virus control (no IFN- γ , dotted line) were included in the test.

detected. The total absence of Ala (which is located at positions 123 and 124) excluded Lys¹²⁵ as the C-terminal residue. Instead, small amounts of Gly and Thr suggested Lys¹²⁸ as the main C-terminal residue, which confirmed the MALDI-MS result. This fragment is referred to below as IFN- γ Δ 15. A second minor fragment (less than 10%) is Met⁰-Arg¹²⁹, as indicated by both the MALDI-MS and C-terminal amino acid analyses.

The IFN- γ C-terminal amino acid sequence is shown below; the bonds cleaved by the different enzymes are indicated by the superscript abbreviations Ch (chymotrypsin), Cl (clostripain) and Pl (plasmin):
-Pro¹²²-Ala-Ala-Lys-Thr-Gly-Lys^{Pl}-Arg^{Cl}-Lys-Arg-Ser-Gln-Met-Leu-Phe^{Ch}-Arg-Gly-Arg-Arg-Ala-Ser-Gln¹⁴³.

Receptor-binding capacity of intact IFN- γ and its derivatives IFN- γ Δ 7, IFN- γ Δ 14 and IFN- γ Δ 15

An initial comparison between intact IFN- γ and its C-terminally shortened fragments was performed by means of a competition ELISA, in which the IFN- γ variants were allowed to compete with immobilized IFN- γ for binding to soluble ECR (Figure 4, upper panel). The identical nature of the curves obtained for the

Table 1 Kinetic rate constants for the interaction between immobilized ECR and various IFN- γ preparations

Interaction with	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{diss} (s^{-1})	K_{a} (M^{-1})
IFN- γ	6.1×10^5	1.6×10^{-3}	3.8×10^8
IFN- γ Δ 7	8.2×10^5	1.65×10^{-3}	5.0×10^8
IFN- γ Δ 14	9.3×10^4	5.8×10^{-3}	1.6×10^7
IFN- γ Δ 15	—*	—*	—*

* The interaction between IFN- γ Δ 15 and ECR was too weak to be analysed ($K_{\text{a}} < 10^5 \text{ M}^{-1}$).

intact IFN- γ and for IFN- γ Δ 7 indicates that the last seven residues are not involved in receptor binding. Another competition ELISA, in which IFN- γ Δ 7 was coated on the surface of the wells, resulted in exactly the same curves (results not shown). This observation is consistent with our knowledge of the structure of natural IFN- γ at its C-terminal region: even though the C-terminal tail of biologically active natural IFN- γ is heterogeneous [5] with the longest polypeptide lacking five residues [6], the removal of short amino acid sequences need not hamper biological activity.

The additional removal of another seven residues (IFN- γ Δ 14), however, significantly decreased the ECR binding capacity of the molecule: the IC₅₀ for IFN- γ Δ 14 was 11-fold higher than for both intact IFN- γ and IFN- γ Δ 7. This observation indicates the interaction of at least a part of the deleted Lys¹³⁰-Phe¹³⁶ sequence with the IFN- γ receptor. In particular, it is believed that Lys¹³⁰, Arg¹³¹ [11] and Ser¹³² [10] are involved in receptor binding. The further omission of only one residue, Arg¹²⁹, markedly decreased the capacity of IFN- γ to interact with ECR, i.e. an IFN- γ Δ 15 concentration of 362 nM was insufficient to decrease the A₄₀₅ to 50%. Moreover, the small fraction (less than 10%) of the IFN- γ Δ 14 polypeptide present in this preparation might account for the slight decrease that was observed at the highest concentration investigated.

Titration of the IFN- γ variants in an antiviral assay on A549 cells showed the same trend (Figure 4, lower panel). However, the ability of IFN- γ Δ 14 to induce an antiviral state was decreased to as little as 1/42 of that of the intact dimer. The observation that IFN- γ Δ 14 produces antiviral activity at proportionally higher concentrations than needed during the competition ELISA results most probably from the fact that an IFN- γ dimer must simultaneously bind two surface-exposed receptor molecules to trigger signal transduction and a cellular response [2,20,21]. Consequently the decreased capacity of a truncated IFN- γ variant in binding to its receptor is more pronounced in the bioassay. The IFN- γ Δ 15 variant did not exhibit any biological activity.

Finally, the IFN- γ -ECR interaction was investigated by measurements of surface plasmon resonance on a BIAcore® instrument. The binding of IFN- γ and its derivatives could be fitted to a simple monovalent interaction model: the association and dissociation rate constants calculated for this binding are listed in Table 1. Whereas the difference in K_{a} values for IFN- γ and IFN- γ Δ 7 is due solely to a slightly different association rate (k_{ass}), the K_{a} value for IFN- γ Δ 14 is only $1.6 \times 10^7 \text{ M}^{-1}$, which is 1/24 of that of the intact molecule. Again, the absence of Arg¹²⁹ (IFN- γ Δ 15) led to a marked decrease in affinity.

These observations clearly demonstrate that Arg¹²⁹ is of crucial importance for the binding of IFN- γ to recombinant ECR and hence to the simultaneous binding to two cellular receptors.

Truncation of the C-terminal region of IFN- γ beyond Arg¹²⁹ resulted in a biologically inactive dimer. Our results differ from that suggested by the crystal structure of the IFN- γ /receptor complex determined by Walter et al. [3], in which the C-terminal sequence Pro¹²²–Gly¹³⁸ of IFN- γ extends away from the receptor into the solvent. However, it was also suggested by those authors that a rotation of 95° about Ala¹²⁴ places the basic residues of the C-terminal sequence in such a position that they can interact with an acidic cluster in the receptor. A comparison of our results with those obtained by others [7–11,22] strongly indicates that a particular sequence in the C-terminal region of IFN- γ , namely the tetrapeptide Arg¹²⁹–Lys–Arg–Ser¹³², greatly contributes to the high-affinity interaction with its receptor and consequently in the capacity of IFN- γ to trigger multiple target cell responses.

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REFERENCES

- Farrar, M. A. and Schreiber, R. D. (1993) *Annu. Rev. Immunol.* **11**, 571–611
- Fountoulakis, M., Zulauf, M., Lustig, A. and Garotta, G. (1992) *Eur. J. Biochem.* **208**, 781–787
- Walter, M. R., Windsor, W. T., Nagabhushan, T. L., Lundell, D. J., Lunn, C. A., Zauodny, P. J. and Narula, S. K. (1995) *Nature (London)* **376**, 230–235
- Grzesiek, S., Döbeli, H., Gentz, R., Garotta, G., Labhardt, A. M. and Bax, A. (1992) *Biochemistry* **31**, 8180–8190
- Rinderknecht, E., O'Connor, B. H. and Rodriguez, H. (1984) *J. Biol. Chem.* **259**, 6790–6797
- Pan, Y.-C. E., Stern, A. S., Familletti, P. C., Khan, F. R. and Chizzonite, R. (1987) *Eur. J. Biochem.* **166**, 145–149
- De La Maza, L. M., Peterson, E. M., Burton, L. E., Gray, P. W., Rinderknecht, E. and Czarniecki, C. W. (1987) *Infect. Immun.* **55**, 2727–2733
- Slodowski, O., Böhm, J., Schöne, B. and Otto, B. (1991) *Eur. J. Biochem.* **202**, 1133–1140
- Döbeli, H., Gents, R., Jucker, W., Garotta, G., Hartmann, D. W. and Hochuli, E. (1988) *J. Biotechnol.* **7**, 199–216
- Lundell, D., Lunn, C., Dalgarno, D., Fossetta, J., Greenberg, R., Reim, R., Grace, M. and Narula, S. (1991) *Protein Eng.* **4**, 335–341
- Schein, C. H. and Haugg, M. (1995) *Biochem. J.* **307**, 123–127
- Haelewyn, J. and De Ley, M. (1995) *Biochem. Mol. Biol. Int.* **37**, 1163–1171
- Leinikki, P. O., Calderon, J., Luquette, M. H. and Schreiber, R. D. (1987) *J. Immunol.* **139**, 3360–3366
- Yokoyama, S., Oobayashi, A., Tanabe, O. and Ichishima, E. (1975) *Biochim. Biophys. Acta* **397**, 443–448
- Giard, D. J., Aaronson, S. A., Todaro, G. J., Arnstein, P., Kersey, J. H., Dosik, H. and Parks, W. P. (1973) *J. Natl. Cancer Inst.* **51**, 1417–1423
- Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfås, S., Persson, B., Roos, H., Rönnerberg, I. et al. (1991) *BioTechniques* **11**, 620–627
- Horvat, R. T. and Parmely, M. J. (1988) *Infect. Immun.* **56**, 2925–2932
- Arakawa, T., Horan, T. P., McGinley, M. and Rohde, M. F. (1990) *J. Interferon Res.* **10**, 321–329
- Hogrefe, H. H., McPhie, P., Bekisz, J. B., Enterline, J. C., Dyer, D., Webb, D. S. A., Gerrard, T. L. and Zoon, K. C. (1989) *J. Biol. Chem.* **264**, 12179–12186
- Greenlund, A. C., Schreiber, R., Goeddel, D. and Pennica, D. (1993) *J. Biol. Chem.* **268**, 18103–18110
- Kotenko, S. V., Izotova, L. S., Pollack, B. P., Mariano, T. M., Donnelly, R. J., Muthukumaran, G., Cook, J. R., Garotta, G., Silvennoinen, O., Ihle, J. N. and Pestka, S. (1995) *J. Biol. Chem.* **270**, 20915–20921
- Seelig, G. F., Wijdenes, J., Nagabhushan, T. L. and Trotta, P. P. (1988) *Biochemistry* **27**, 1981–1987