The Heparan Sulfate Binding Sequence of Interferon-γ Increased the On Rate of the Interferon-γ-Interferon-γ Receptor Complex Formation*

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Interferon-γ (IFNγ), in common with a number of growth factors, binds both to heparan sulfate or heparin-related molecules and to a specific high affinity receptor (IFNγR). Using surface plasmon resonance technology, kinetic analysis of the IFNγ-IFNγR complex formation was performed with the extracellular part of IFNγR immobilized on a sensor chip. At the sensor chip surface, IFNγ was bound by two IFNγR molecules with an affinity in the nanomolar range (0.68 nM). This binding was characterized by an important on rate, $k_{on} = 7.3 \times 10^7 \text{M}^{-1}\text{s}^{-1}$, and an off rate, $k_{off} = 5 \times 10^{-3}\text{s}^{-1}$. This binding assay was used to investigate a possible role of heparin in the IFNγ-IFNγR complex formation. In contrast to growth factors for which binding to heparin is usually required for high affinity receptor interaction, we found in this study that IFNγ bound to heparin displayed a strongly reduced affinity for its receptor. This is consistent with the fact that a cluster of basic amino acids (KGGKRKR, called the C1 domain) in the carboxyl-terminal sequence of the cytokine was involved both in heparin and receptor recognition. To understand how a single domain of IFNγ could be implicated in two discrete functions (i.e. binding to heparin and to IFNγR), we also analyzed in a detailed manner the role of the IFNγ carboxyl-terminal sequence in receptor binding. Using forms of IFNγ, with carboxyl terminus truncations of defined regions of the heparin binding sequence, we found that the C1 domain functioned by increasing the on rate of the IFNγ-IFNγR binding reaction but was not otherwise required for the stability of the complex. Interactions between the IFNγ carboxyl-terminal domain and IFNγR could increase the association rate of the reaction either by increasing the number of encounters between the two molecules or by favoring productive collisions. The mechanisms by which heparan sulfate regulates IFNγ activity may thus include both control of selective protease cleavage events, which directly affect the cytokine activity, and also an ability to modulate the interaction of IFNγ with the IFNγR via competitive binding to the C1 domain.

Interferon-γ (IFNγ) is a highly pleiotropic protein secreted by activated T-lymphocytes and natural killer cells (1). The active form of this cytokine is a homodimer consisting of two intertwined 143-amino acid polypeptides. Each monomer consists of six helices (denoted A–F), linked by loops, and an unfolded sequence at the carboxyl-terminal side (downstream to the F helix, amino acids 124–143), which extends away from the molecule (2–4). IFNγ mediates its pleiotropic activities through a specific transmembrane receptor (IFNγR) expressed at the surface of almost all cells (5). This receptor is composed of a ligand-binding subunit, or α-chain (IFNγRa), and an accessory factor, or β-chain which is required for signal transduction (6, 7). The IFNγIFNγRα complex consists of two receptors bound to an IFNγ dimer, a stoichiometry consistent with the symmetry of the ligand (8, 9). IFNγ residues involved in receptor binding are situated in two areas of the molecule (10, 11): the loop connecting the A and B helices (residues 18–26) and the helix F (residues 108–124). The carboxyl-terminal sequence (residues 124–143) is highly flexible and/or adopts multiple conformations (3, 4). It contains two small clusters of basic amino acids (C1, residues 125–131, KGGKRKR; C2, residues 137–140, RGRR), which confer on the molecule a important sensitivity to a variety of proteases (12–15). As a consequence, native IFNγ is usually a mixture of carboxyl-truncated molecules lacking up to 16 amino acids and therefore may end anywhere from Gly143 (full-length molecule) to Gly127. Since deletion of less than 10 amino acid increases the bioactivity of IFNγ, while more extended cleavages of the carboxyl-terminal domain have the opposite effect, it has been speculated that the cytokine activity can be modulated by limited proteolysis. The portion of the carboxyl terminus that appeared to be the most important in this process is the basic sequence KRR of the C1 domain, since the beginning of its removal correlated with loss of activity, while cleavages downstream of this sequence resulted in the observed increased activity (13). However, as revealed by the crystal structure of the IFNγ-IFNγRα complex, specific interactions between this basic sequence of IFNγ and IFNγRα have not been identified, and the carboxyl-terminal tail of the cytokine did not appear to be involved in receptor binding (11). Furthermore, while both antibodies directed against amino or carboxyl terminus block biological activity, only synthetic peptides comprising the amino-terminal domain of the cytokine block binding to its receptor in a competitive manner (15). In addition, it has been shown that if a positive charge localized in the carboxyl-terminal part of the cytokine is impor-

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The abbreviations used are: IFNγ, interferon-γ; IFNγR, interferon-γ receptor; IFNγRα, IFNγ receptor α-chain; FGF, fibroblast growth factor; mAb, monoclonal antibody; RU, resonance units; sIFNγR, soluble IFNγ receptor.
 tant for activity, it can be contributed by a number of different combinations of positions. Therefore, this part of the molecule did not appear to function in a specific manner, and its function remains unclear (16). The carboxyl-terminal domain of IFNγ also confers on the molecule a high affinity ($K_d = 1.5$ nM) for heparan sulfate (17) or heparin-related molecules (18). A fragment of heparan sulfate that displays high affinity for IFNγ has been isolated. It consists of two small sulfated heparin-like sequences linked together by an extended internal N-acetylated rich domain (19). In the IFNγ-heparan sulfate complex, the two carboxyl termini of an IFNγ dimer interacted with the two heparin-like sequences of the heparan sulfate fragment, mainly through the basic C1 and C2 domains (20).

In this study, we investigated whether or not IFNγ bound to heparin could also interact with its high affinity cell surface receptor. Using the Biacore system, we analyzed kinetic aspects of the IFNγ-IFNγR complex formation and, in particular, the importance of the heparin binding sequence of IFNγ for this interaction. We found that IFNγ binding to heparin and to IFNγR are mutually exclusive and that the C1 sequence increased the association rate of the IFNγ-IFNγR binding reaction.

MATERIALS AND METHODS

**Equipment and Reagents**—An upgraded Biacore system, certified CM5 sensor chips, and HBS buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.05% surfactant P20, pH 7.4) were from Biacore AB. Biotin- LC-hydrazide was from Pierce, and streptavidin was from Sigma. Proteolytic enzymes (activated factor X, carboxypeptidase Y, endoproteinase Arg-C) and enzyme inhibitor (pefabloc) were from Boehringer. Recombinant IFNγ (IFNγ145, batch number L 405), IFNγ lacking the last 19 amino acids on the carboxy-terminal side (IFNγ126), and the two monoclonal antibodies (283-4-45 and 13-16-2) used in this study were kind gifts of Roussel Uclaf company. The extracellular part of the human IFNγ receptor (sIFNγR- α-chain, produced in SF9 cells, was a kind gift of Dr. L. Ozmen (Hoffmann La Roche), and fractionated heparin was from Sanofi Recherche.

**Preparation of IFNγ Lacking Carboxy-terminal Amino Acids and the IFNγ Assay**—Full-length IFNγ (IFNγ145) was digested with either activated factor X (1 unit/ml in 50 mM Tris, 150 mM NaCl, pH 8, buffer for 1 h at 37 °C), carboxypeptidase Y (1 unit/ml in 50 mM Tris, 150 mM NaCl, pH 8, buffer for 4 h at 25 °C). Enzymatic reactions were stopped with 1 mM pefabloc and analyzed using 15% SDS-polyacrylamide gel electrophoresis and by electrospray ionization mass spectrometry. For that purpose, digested samples were made 0.4 mg/ml in 10 mM ammonium acetate buffer and analyzed using a Perkin-Elmer Sciex API III triple quadrupole mass spectrometer equipped with a 35°-70° electrospray-assisted electrospray (ionspray) source. Samples were directly infused into the source using a syringe pump at a flow rate of 5 μl/min and an injector equipped with a 1-μl internal loop or were desalted using a reversed phase high pressure liquid chromatography column coupled to the electrospray source. The ionspray probe tip was held at 5000 V, and spectra were recorded in the 900–1800 range of mass-to-charge ratios in steps of 0.4 m/z, with a 1.7-s dwell time. The signal was averaged over four scans. Specific antiviral activity of the IFNγ samples were determined with a microtiter inhibition of cytopathic effect assay using Wisch cells against vesicular stomatitis virus (21).

**Biotinylation of the sIFNγR**—The purified sIFNγR (22) was prepared at 0.27 mg/ml (8 μM) in 20 mM phosphate buffer, pH 7, and reacted for 20 min in the dark and at 4 °C with 10 mM sodium periodate to oxidize the glycan parts of the molecule. The reaction was quenched with 15 mM glycerol, and the sample was dialyzed against the 20 mM phosphate buffer. Biotin-hydrazide (including a 2.5-nm spacer arm between the biotin and hydrazide groups) was then added to a concentration of 5 mM, and the mixture was incubated for 6 h at 4 °C. The sample was made to 0.1 mM ethanolamine, and then extensively dialyzed against phosphate-buffered saline, pH 7.2. Biotinylation was checked by Western blot analysis, and samples were aliquoted and stored at −80 °C.

**Preparation of the Biacore Binding Surface**—The flow rate of the running buffer (HBS) was maintained at 5 μl/min, and the temperature was maintained at 25 °C. Two flow cells of a CM5 sensor chip were activated with 50 μl of a mixture of 0.2 M 1-ethyl-3-(3 dimethylamino propyl) carbodiimide 0.05 M N-hydroxysuccinimide, after which 50 μl of streptavidin (0.2 mg/ml in 10 mM acetate buffer, pH 4.2) was injected. Unreacted groups were blocked with a 50-μl injection of 1 M ethanolamine, pH 8.5. Approximately 8000 resonance units (RU) of streptavidin were fixed on the surface by this procedure. Biotinylated sIFNγR (10 μl/mg in HBS) was then injected for 1 min on one of the two flow cells, while the other was a negative control. Both flow cells were then conditioned with 10 2-min pulses of 10 mM HCl. This resulted in the attachment of 800 RU of IFNγR. The conversion of RU to surface concentration of proteins was performed using a conversion factor of 1000 RU = 1 ng/mm².

**Binding Assay**—Test samples were diluted in HBS maintained at 25 °C and injected over the IFNγR surface at a flow rate of 50 μl/min. This high flow rate was necessary to reduce mass transport effect due to the high association rate of the proteins being studied. Using the Kinject command, usually 200 μl of IFNγ or IFNγ-derived molecules were injected across the IFNγR surface, after which the formed complexes were washed at 50 μl/min with HBS to study the dissociation phase. The IFNγR surface was regenerated with a 2-min pulse of 10 mM HCl. For kinetic analysis, a complete set of sensograms were recorded with eight different IFNγ concentrations in the range 0–0.5 μg/ml.

**Kinetic Analysis**—Sets of sensograms were analyzed with the Biacore 2.1 software, provided with the machine, using both linear transformation of the primary data and nonlinear fitting of the sensorgrams. Brieferly, the equation for the measured binding rate (dR/dt) as a function of the binding response (R) is dR/dt = k₅ [IFNγ] + k₄ [IFNγ]. Where $k₅$ and $k₄$ are the association and dissociation rate constants. C is the concentration of the analyte (IFNγ). $R_{max}$ is the binding capacity of the immobilized ligand (IFNγ-R), and $R_{t}$ is the amount of analyte bound to ligand at time $t$. Kinetic constants ($k₅$ and $k₄$) can be obtained by linear transformation of a set of sensograms using a plot of ln(dR/dt) versus time for each analyte concentration. These plots give a line of which the slope is $k₅$. A secondary plot of these slopes ($k₅$ versus C is then used to determine $k₅$ and $k₄$ from the linear relationship $k₅$ = $k₄$C + $k₆$. Association and dissociation rate constants can also be extracted from a single sensogram (i.e. a single analyte injection). In that case, the integrated forms of the rate equations ($r_{on}$ = $R_{max}$ $exp(-(k₄$ $t)/$D$)$ for dissociation and $r_{off}$ = $R_{max}$ ($1 - k₅$ $R_{max}$ $t$) for association, where $R_{max}$ is the steady-state binding response and $t$ and $t₀$ are the start times for association and dissociation) were fitted to the experimental data by nonlinear regression (23). In some cases to derive kinetic constants, numerical integration, which allows fitting of data to complex interaction models, was performed (Biavalue 3.0 software). Data were analyzed by global fitting of both association and dissociation phases for several concentrations simultaneously (24). Affinity (association equilibrium constants, $Kₐ$) were calculated from the ratio of dissociation and association rate constants ($Kₐ$ = $k₆$/d$k₅$).

**RESULTS**

**Binding of IFNγ to Sensor Chip Immobilized IFNγR**—Using the Biacore technology, we set up a binding assay to analyze the IFNγ-IFNγR interaction, in particular the kinetic aspects. The Biacore instrument uses surface plasmon resonance to measure changes in refractive index when a soluble analyte (here IFNγ) binds to an immobilized ligand (IFNγ-R). Biotinylated sIFNγR was immobilized on a streptavidin-activated sensor chip, at a density of 0.8 ng/mm² (800 RU). Biotin groups had been attached to the glycan part of the receptor (a part not required for ligand binding) to ensure that 100% of the molecules remain active upon immobilization. When IFNγ was injected over the IFNγR surface, a typical sensogram was obtained (Fig. 1a), with an association phase (A), equilibrium (E), and, when IFNγ was replaced by running buffer alone, a dissociation phase (D). Preincubation of the cytokine with increasing concentrations of its soluble receptor completely inhibited the interaction with the immobilized IFNγ-R, demonstrating the specificity of the binding (Fig. 1b). In addition, upon injection of IFNγ over the surface (containing streptavidin only) no binding was observed (Fig. 1b).

Maximum binding was approximately 400 RU. Since we had 800 RU of immobilized IFNγ-R, this suggests that each IFNγ dimer (34 kDa) has been bound by two IFNγ-R molecules (32 kDa each). Dimerization of IFNγ-R by its ligand also occurs both in solution and at the cell surface (9). To further confirmed this...
soluble IFN showed that the binding in association phase (A), the equilibrium (E), and the dissociation phase (D). When coincubated with soluble IFN-R, the binding of IFNγ was strongly reduced. b, IFNγ (eight concentrations from 0 to 0.5 μg/ml) was injected on a streptavidin-activated sensor chip, which showed that the binding in a was specific to the immobilized IFNγ-R. c, IFNγ (0.5 μg/ml) was injected on the IFNγ-R-activated sensor chip until the equilibrium was reached. After a quick wash with running buffer, soluble IFNγ-R (1, 2, 10, 20 μg/ml, from top to bottom) was injected on the bound IFNγ. The soluble IFNγ-R could not bind to a preformed IFNγ-IFNγ-R complex, demonstrating that at the surface of the sensor chip the cytokine was already bound by two IFNγ-R molecules.

FIG. 1. IFNγ binding to immobilized IFNγ-R. a, IFNγ (0.5 μg/ml), either alone or in combination with 2.5, 5, 10, or 20 μg/ml soluble IFNγ-R, was injected for 4 min (from 130 to 370 s) to an IFNγ-R activated sensor chip, after which the samples were replaced by running buffer. The binding response in RU was recorded as a function of time and showed the association phase (A), the equilibrium (E), and the dissociation phase (D). When coincubated with soluble IFNγ-R, the binding of IFNγ was strongly reduced. b, IFNγ (eight concentrations from 0 to 0.5 μg/ml) was injected on a streptavidin-activated sensor chip, which showed that the binding in a was specific to the immobilized IFNγ-R. c, IFNγ (0.5 μg/ml) was injected on the IFNγ-R-activated sensor chip until the equilibrium was reached. After a quick wash with running buffer, soluble IFNγ-R (1, 2, 10, 20 μg/ml, from top to bottom) was injected on the bound IFNγ. The soluble IFNγ-R could not bind to a preformed IFNγ-IFNγ-R complex, demonstrating that at the surface of the sensor chip the cytokine was already bound by two IFNγ-R molecules.

FIG. 2. IFNγ bound to heparin displayed a reduced binding to IFNγ-R. IFNγ (0.5 μg/ml) was preincubated with 0.5-, 5-, 10-, or 50-fold molar excess (from top to bottom on each set of sensogram) of different heparin molecules. Bound to the 12.5-kDa heparin molecule (a), IFNγ displayed a strongly reduced binding to IFNγ-R. Heparin fragments of 4.5 (b) or 1.8 kDa (c) had a reduced ability to inhibit the IFNγ binding to IFNγ-R.
with the known substrate specificity of the enzymes used. These molecules therefore ended at Arg\textsuperscript{157}, Gln\textsuperscript{158}, and Arg\textsuperscript{129} and will be referred to as IFN\textsubscript{Y137}, IFN\textsubscript{Y138}, and IFN\textsubscript{Y129} (see Fig. 4). As a control, IFN\textsubscript{143} was also analyzed, and the \( M_r \) obtained (16,908) was exactly the \( M_r \) expected for the full-length molecule. In addition, deletion of six (IFN\textsubscript{Y137}) or 10 (IFN\textsubscript{Y133}) amino acids resulted in the expected increased antiviral activity (13), while deletion of 14 (IFN\textsubscript{Y129}) or 19 (IFN\textsubscript{Y124}) amino acids resulted in the expected decreased activity for such truncated molecules (data not shown).

**Kinetic Analysis of the Interaction between IFN\textgamma and IFN\gammaR:**

*Role of the Carboxyl-terminal Sequence—IFN\textsubscript{143} (full-length IFN\textgamma), and the carboxyl termini-deleted IFN\textgamma were injected over the biacore IFN\gammaR surface, each with a range of concentrations to produce sets of sensorgrams from which association and dissociation phases can be analyzed. The dissociation phase started at 370 s, when the injection of IFN\gamma was changed to a perfusion buffer. To this part of the sensorgrams was applied a simple \( AB = A + B \) dissociation model. A good fit was found for IFN\textsubscript{Y124} only (Fig. 5c), which gave an off rate in the range 4.7–5.2 \( \times 10^{-3} \mathrm{s}^{-1} \). For the others, the higher the observed on rate (see below) the worse was the fit. Presumably, the fast on rate caused a rapid rebinding of the dissociated molecules, a phenomenon that occurs as increasing numbers of free immobilized ligand (IFN\gammaR) are regenerated at the surface of the sensor chip during the dissociation phase. Since free IFN\gammaR, and therefore the rebinding effect, increased with time during the dissociation, only the first 60 s were used for the analysis. In addition, to prevent rebinding of dissociated molecules, soluble IFN\gammaR was included in the perfusion buffer during dissociation (e.g. see Fig. 1c). Including IFN\gammaR at 10–20 \( \mu \mathrm{g/ml} \) in the perfusion buffer, a simple dissociation model can now be assumed, characterized by an off rate of 5–5.6 \( \times 10^{-3} \mathrm{s}^{-1} \). Interestingly, this \( k_{\text{off}} \) was similar to the \( k_{\text{off}} \) of IFN\textsubscript{Y124} (for which no rebinding occurred). Therefore, the addition of soluble IFN\gammaR in the perfusion buffer was effective in preventing rebinding and necessary for accurate kinetic analysis. Furthermore, these data showed that cleavages of the carboxyl terminus of IFN\gamma did not modify the off rate of the reaction and, thus, that the carboxyl-terminal sequence of

**FIG. 4. Enzymatic cleavage of IFN\gamma**

IFN\gamma was treated with activated factor X, carboxypeptidase Y, or endoproteinase Arg-C. Reactions were stopped with 1 \( \times \) protamine sulfate, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis. Lane A, molecular weight markers (97,400, 66,200, 45,000, 31,000, 21,500, 17,000, and 14,400). Lane B, IFN\textsubscript{Y143}. Lane C, activated factor X-treated IFN\gamma. Lane D, carboxypeptidase Y-treated IFN\gamma. Lane E, endoproteinase Arg-C-treated IFN\gamma. Lane F, IFN\textsubscript{Y133}. Lane G, a mixture of lanes B–F. The carboxyl-terminal sequence of IFN\gamma is also shown. The two clusters of basic amino acids C1 and C2 are in boldface type, and the cleavage sites, as determined by electrospray ionization mass spectrometry (see Table I), are indicated by arrows.

**FIG. 3. The C1, but not the C2, domain of IFN\gamma is involved in receptor binding.** IFN\gamma (0.5 \( \mu \mathrm{g/ml} \)) was preincubated with 25, 50, 100, or 200 \( \mu \mathrm{g/ml} \) mAb 293-4-45 (a) or mAb 13-16-2 (b) and then injected over the IFN\gammaR surface. Both mAbs reduced the binding of IFN\gamma to its receptor, the 293-4-45 (which defines the C1 domain) being much more active than the 13-16-2 (which defines the C2 domain). These two mAbs (both at 100 \( \mu \mathrm{g/ml} \)) were also injected on a preformed IFN\gammaR complex (c). mAb 13-16-2 could bind to the IFN\gammaR complex, whereas mAb 293-4-45 only displayed a weak interaction.
Role of the IFNγ Carboxyl Terminus in IFNγ-IFNγR Binding

Full-length IFNγ (IFNγ143), activated factor X (aFX)-, carboxypeptidase Y (CY)-, or endoproteinase Arg-C (ArgC)-treated IFNγ was analyzed by electrospray ionization mass spectrometry. Cleavage points (last amino acid) were determined from the measured relative molecular mass (M_r).

| Treatment | aFX | CY | ArgC |
|-----------|-----|----|------|
| Last amino acid | IFNγ143 | Gln143 | 16908 ± 2 |
| Name | IFNγ137 | Arg137 | 16254 ± 2 |
| | IFNγ133 | Gln133 | 15705 ± 2 |
| | IFNγ129 | Arg129 | 15208 ± 2 |
| | IFNγ124 | Ala124 | ND* |

*Not determined.

**TABLE I**

Cleavage points of the IFNγ truncated forms

**FIG. 5.** Overlay of sensorgrams showing binding of IFNγ to immobilized IFNγ-R. Role of the IFNγ carboxyl-terminal sequence. IFNγ143 (a), IFNγ137 (b), IFNγ133 (c), IFNγ132 (d), and IFNγ124 (e) were injected over an IFNγ-R-activated surface at a flow rate of 50 μl/min during 4 min (from 130 to 370 s), after which running buffer was injected, and the response in RU was recorded as a function of time. Each set of sensorgrams was obtained from the data used for evaluation, as already reported in several other cytokine-receptor binding studies (27, 28). In this case, this was observed (see below) for the molecules that displayed a high on rate (IFNγ143, IFNγ137, and IFNγ133), but not for the others (IFNγ123 and IFNγ124). The second phase was determined by the kinetics of the IFNγ/IFNγR interaction, and the third one was equilibrium. It was immediately apparent (Fig. 5) that the binding rate of the reaction increased for IFNγ137 and IFNγ133, compared with full-length molecule (Fig. 5, a–c) but decreased for IFNγ123 and IFNγ124 (Fig. 5, d–e). Kinetic analysis of the binding curves was first performed with the Biaevaluation 2.1 software. As indicated above, in some cases (IFNγ143, IFNγ137, and IFNγ133), the early phase of the association was dominated by mass transport. However, as the reaction proceeds, the free binding sites decrease and the binding rate progressively depends on the intrinsic rate constant. Thus, as a first approach, when analyzing the experiments, the mass transport-limited parts of the sensorgrams were excluded from the data used for evaluation, as already reported in several other cytokine-receptor binding studies (27, 28). In this way, the kinetic part of each sensorgram could be confidently fitted on the basis of an A + B = AB model, and deviations of the data points from the fitted curve were quantitatively in the range expected from the background noise. As detailed under "Materials and Methods," analysis was performed both by nonlinear fitting and by linear transformation of the data. Both methods gave similar kinetic constants, and the results are summarized in Table II.

However, mass transport limitation has been identified as a potential problem, leading to underestimation of the association rate constant (29). Thus, to better assess the observed changes in on rates, caused by cleavages of the IFNγ carboxyl-terminal sequence, we also analyzed our data by numerical integration, using the Biaevaluation 3.0 software. Here, in contrast to the previous analysis, the entire time courses of the reactions were fitted to binding models, including mass transport-limited binding reaction. Global fitting of binding curves, recorded with IFNγ129 and IFNγ124, gave respective association rate constants of 2.48 and 1.9 × 10^6 M^-1 s^-1. It is noteworthy that these values are in close agreement with those determined either by linear transformation of the data or by nonlinear fitting (Table II). In contrast, global fitting of the binding curves obtained with IFNγ143, IFNγ137, and IFNγ133 was only possible when mass transport limitation was introduced in the model. Data are reported in Table II and showed that these three molecules bound IFNγ-R with respective on rate constants of 0.73, 1.3, and 1.57 × 10^7 M^-1 s^-1. These values are, on average, twice as much as those determined with the first approach and showed that k_on values were underestimated when only parts of the sensorgrams were used for the...
Role of the IFN-γ Carboxyl Terminus in IFN-γR Binding

TABLE II

| Signaling event | k_{off} | k_{on(a)} | k_{on(b)} | k_{on(c)} | k_{on} |
|-----------------|---------|-----------|-----------|-----------|--------|
| IFN-γR         | 5.4 × 10^{6} | 4.6 × 10^{6} | 7.3 × 10^{6} | 1.0 × 10^{6} | 0.68   |
| IFN-γR         | 8.8 × 10^{6} | 7.2 × 10^{6} | 1.3 × 10^{6} | 3.8 × 10^{6} | 0.38   |
| IFN-γR         | 6.9 × 10^{3} | 6.2 × 10^{3} | 1.57 × 10^{3} | 3.2 × 10^{3} | 0.32   |
| IFN-γR         | 2.4 × 10^{3} | 2.1 × 10^{3} | 2.48 × 10^{3} | 2.00 × 10^{3} | 2.00   |
| IFN-γR         | 1.8 × 10^{3} | 1.4 × 10^{3} | 1.9 × 10^{3} | 2.63 × 10^{3} | 2.63   |

fitting procedure.

Together these analyses showed that progressive cleavages of the IFN-γ carboxyl terminus progressively increased the on rate of the binding to IFN-γR until the C1 domain was affected. The maximum on rate was observed with IFN-γR binding cytokine. which was caused by a reduction of the on rate of the reaction (5.3, 9.3). Using this two molecules are bound to each other (11). Attracting forces between these two charged domains could place the two interacting molecules into a proper orientation while they are still some distance apart. In such a way, the specific binding sites may collide with each other more frequently, and this would result in the increased on rate observed when C1 was present in the molecule (IFN-γR, IFN-γC2, and IFN-γC1). Another mechanism could be proposed whereby interacting IFN-γ and IFN-γR are held together by relatively nonspecific forces, (between C1 and the group of acidic amino acids found in IFN-γR) long enough to increase their chance of finding a mutually reactive configuration. A mechanism of this sort can be viewed as representing reorientation within an encounter complex (44) and may account for the importance of C1 during the association phase only. It is noteworthy that the association rate can be increased for a variety of molecules, simply by attaching weakly interacting, relatively unstructured polymeric domains to the macromolecules involved. Furthermore, charged residues are particularly suitable for such mechanisms, because electric fields that surround such groups spread out in every direction, making collision geometry less important (44). Together, our data and the proposed mechanisms described above suggest that C1 interaction with IFN-γR is the first event of the IFN-γR binding complex formation. The dissociation rate was identical for all of the IFN-γ forms studied here, irrespective of the presence of C1 and C2 domain (k_{off} = 5 × 10^{-3} s^{-1}). However, for those forms that contained the C1 region, it was necessary to include soluble IFN-γR in the running buffer during the dissociation phase to prevent rebinding of dissociated molecules. The observation that the C1 sequence caused immediate rebinding of newly dissociated molecules further supports the idea that interaction between C1 and IFN-γR represents the first contact between the two molecules.
For analytical purposes, rebinding has been eliminated in our study, but this may have a physiological importance. IFNγ is active at extremely low concentration, well below the $K_d$ (for example, a few pM only are required to have an antiviral effect in cell culture). Therefore, free IFNγR is in large excess, and rebinding of dissociated molecules is likely to occur at the cell surface. As a result, the apparent affinity of IFNγ for its receptor will be increased (see result from Fig. 1a). High affinity, and in particular a high rate of association, is necessary for achieving a high rate of product formation but also for preventing side reactions such as inactivation.

High affinity binding of IFNγ to heparin also represents a mechanism by which IFNγ is protected from inactivation (20). This binding also supports the plasma clearance of the cytokine (46). It is noteworthy that these effects of heparin or heparan sulfate on IFNγ activities did not depend on IFNγR, and this is consistent with the fact that binding to heparin and IFNγR are two independent events. However, since IFNγ cannot bind heparin and its receptor simultaneously, it remains unclear how the heparin-bound cytokine can be released to interact with its receptor, and this point will be the subject of future studies. Finally, the results of the present work together with our previous reports clearly indicate that IFNγ and growth factors (such as FGF) belong to distinct groups regarding their regulation by heparin/heparan-like molecules.

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