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BINDING OF IFNγ AND ITS C-TERMINAL PEPTIDE TO A CYTOPLASMIC DOMAIN OF ITS RECEPTOR THAT IS ESSENTIAL FOR FUNCTION

Brian E. Szente* and Howard M. Johnson

Departments of Microbiology and Cell Science and Pathology, The University of Florida, Gainesville, FL 32611

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Summary: We have previously shown that murine interferon gamma (IFNγ) binds to a soluble form of its receptor via both the N-terminus and C-terminus. The IFNγ N-terminus binds extracellular receptor residues 95-120. Here we report that the C-terminus of IFNγ binds to the membrane proximal region of the cytoplasmic domain of the receptor, residues 253-287. Peptide binding to fixed/permeabilized cells is specifically blocked by anti-(253-287) antibodies. These data suggest a novel mechanism by which IFNγ binds to its receptor, involving both the extracellular and the intracellular receptor domains. Such a mechanism could have broader implications for the activation of signal transduction pathways by both IFNγ and other cytokines whose receptors are members of the cytokine receptor superfamily.

Interferon-γ (IFNγ) is a pleiotropic cytokine produced by activated T lymphocytes (subtype Th-1) and natural killer (NK) cells. Among its many effects are the induction of a number of antiviral proteins, upregulation of class II MHC expression, a role in B cell maturation, activation of cells to cytotoxic states, and release of mediators of inflammation (1). These activities are induced as the IFNγ molecule interacts in a species-specific manner with a single class of cell surface receptor and an associated cofactor molecule (2). In both mice and in humans, this receptor is a single chain glycoprotein of approximately 85-90 kD which has fairly large (> 200 amino acids) extracellular and cytoplasmic domains.

We have previously identified the N-terminus and C-terminus of IFNγ as being involved in receptor binding and function using synthetic peptide competition and site-specific antibody neutralization of IFNγ activity (3). The N-terminal IFNγ peptide, IFNγ(1-

* To whom correspondence should be addressed.

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39), specifically bound to receptor in intact membranes and to soluble receptor, while the C-terminal IFNγ peptide, IFNγ(95-133), only bound to the soluble receptor (4). The recombinant soluble receptor consisted of the extracellular and cytoplasmic domains, but lacked the transmembrane sequence (5). Antibodies to both peptides neutralized IFNγ activity, while antibodies corresponding to internal regions of the IFNγ molecule did not neutralize IFNγ (3). C-terminal truncations of IFNγ obtained by recombinant techniques or enzymatic cleavage resulted in loss of biological activity (6-8). In all cases this resulted in removal of a polycationic region of the C-terminus of IFNγ. Thus, these findings indicated that the C-terminus of IFNγ was important for function.

MATERIALS AND METHODS

Synthetic peptides. Overlapping peptides encompassing the intracellular domain of the murine IFNγ receptor were synthesized on a Biosearch 9500AT automated peptide synthesizer using fluorenyl-methyloxy carbonyl (Fmoc) chemistry (9). Peptides were cleaved from the resins using trifluoroacetic acid/ ethanedithiol/ thioanisole/ water at a ratio of 40/1/2/2. The cleaved peptides were then extracted in ether and ethyl acetate and subsequently dissolved in water and lyophilized. Reverse phase HPLC analysis of crude peptides indicated one major peak in each profile. Thus, further purification was unwarranted. Amino acid analysis of these peptides showed that the amino acid composition corresponded closely to theoretical values.

Radioiodinations. IFNγ (1 μg) was radioiodinated by combining 10 μl with 5 μl (500 μCi) $Na^{125}I$ (15 mCi/μg, Amersham) in the presence of 25 μl 0.15 M potassium phosphate buffer, pH 7.4, and 10 μl Chloramine-T (5 mg/ml) for 2 minutes. After neutralization of the reaction with 10 μl volumes of sodium metabisulfite (10 mg/ml), potassium iodide (70 mg/ml), and BSA (20 mg/ml), the preparation was sieved on a 5-ml Sephadex G-10 column. Fractions of 400 μl were collected, and the fraction containing the greatest activity was used for receptor binding studies. The specific activity of ^{125}I-IFNγ was 40-50 μCi/μg of protein.

Biotinylation of IFNγ(95-133). IFNγ(95-133) lacks tyrosine residues, and consequently could not be labelled with ^{125}I. Instead, this peptide was labelled with an excess of NHS-LC-biotin (Pierce) in 50mM bicarbonate buffer (pH=9.6) for 2 hours at 4°C. Unreacted biotin was removed by dialysis.

Binding assays. For solid phase binding assays, receptor peptides were plated in carbonate buffer at a final concentration of 1.5 μg/well in a 96-well microtiter plate. Plates were incubated for 2 hours at room temperature with phosphate buffered saline (PBS) containing 1%BSA and 0.05%NaN_3 in order to block non-specific binding. Following this, ^{125}I-IFNγ was added to the wells at a final concentration of 5nM for a 2 hour incubation. Wells were finally rinsed thoroughly with wash buffer, blotted dry, and counted individually on a Gamma counter. For peptide competition, competitor peptides were incubated in the wells for 2 hours prior to the addition of the ^{125}I-IFNγ.

For binding assays involving biotinylated IFNγ(95-133), receptor peptides were plated and blocked as above, following which 5nM biotinylated IFNγ(95-133) was added to eahc well for a 2 hour incubation. Plates were rinsed with PBS-1%BSA. Next a 1:1000 dilution of streptavidin-alkaline phosphatase conjugate (Sigma) was added for a 90 minute incubation. Plates were rinsed and blotted to dryness. Detection of bound ligand was accomplished by adding 50 μl/well of a 1 mg/ml solution of *p*-nitrophenyl phosphate for 1 hour. Color development was halted by the addition of 50 μl/well of 3M NaOH.

Absorbance was measured at 405 nm in a Microplate Reader (Bio-Rad). For peptide competition, competitor peptides were incubated in the wells for 2 hours prior to the addition of biotinylated IFNγ(95-133).

Binding assayed by flow cytometry. L929 murine fibroblasts were fixed in 0.25% paraformaldehyde for 1 hour at 4°C and then permeabilized in 0.2% Tween-20 for 15 minutes at 37°C. Cells were incubated for 1 hour with either FACS buffer (PBS/0.5% BSA/100 nM NaN_3) or antiserum, washed with FACS buffer, and then incubated with biotinylated peptide IFNγ(95-133) at a final concentration of 100 nM for 1 hour. Cells were washed, and a streptavidin-phycoerythrin conjugate was added. Cells were again washed and finally analyzed on a FACScan (Becton-Dickinson).

RESULTS

Peptides of IFNγ Receptor Cytoplasmic Domain. We have recently shown, using overlapping synthetic peptides, that IFNγ and its N-terminal peptide, IFNγ(1-39), bind to the extracellular domain of the IFNγ receptor via receptor residues 95-120 (10). The IFNγ C-terminal peptide, IFNγ(95-133), did not bind specifically to any of the extracellular receptor peptides. The fact that IFNγ(95-133) bound to the soluble receptor, but not the receptor in intact membranes, suggested the possibility that it binds to a region on the cytoplasmic domain of the IFNγ receptor. Thus, overlapping peptides spanning the entire cytoplasmic domain of the murine IFNγ receptor were synthesized in order to assay solid-phase direct binding of IFNγ and IFNγ(95-133) (Table 1).

Binding of ^{125}I-IFNγ. Only the peptide encompassing the membrane-proximal 35 residues (253-287) significantly bound ^{125}I-IFNγ (Figure 1). A scrambled version of this same peptide (Table 1) was unable to bind IFNγ, thus demonstrating the specificity of this interaction. Unlabelled IFNγ and the C-terminal IFNγ peptide, IFNγ(95-133), competed with ^{125}I-IFNγ for binding to receptor peptide (253-287) (Figure 2). The N-terminal peptide of IFNγ, IFNγ(1-39), whose binding site lies within the receptor's extracellular domain, was unable to compete for this intracellular binding site. As previously shown (4), IFNγ and

Table 1. Sequences of overlapping murine IFNγ intracellular receptor peptides. Peptides were synthesized as described (10). *S denotes a scrambled version of the original sequence and was generated using the sequence edit program (11).

Peptide	Sequence
(253-287)	TKKNSFKRKSIMLPKSLLSVVKSATLETKPESKYS
(283-309)	SKYSLVTPHQPAVLESETVICEEPLS
(305-334)	EEPLSTVTAPDSPEAAEQEELSKETKALEA
(330-360)	KALEAGGSTSAMTPDSPPTPTQRRSFSLLSS
(356-384)	SLLSSNQSGPCSLTAYHSRNGSDSGLVGS
(379-407)	SGLVGSGSSISDLESLPNNNSETKMAEHD
(404-432)	AEHDPPPVRLAPMASGYDKPHMLVDVLVD
(428-452)	DVLVDVGGKESLMGYRLTGEAQELS
(253-287)S*	TSMPLKTVELLRKTKNKYSFKSKSEKKSSLVIPAS

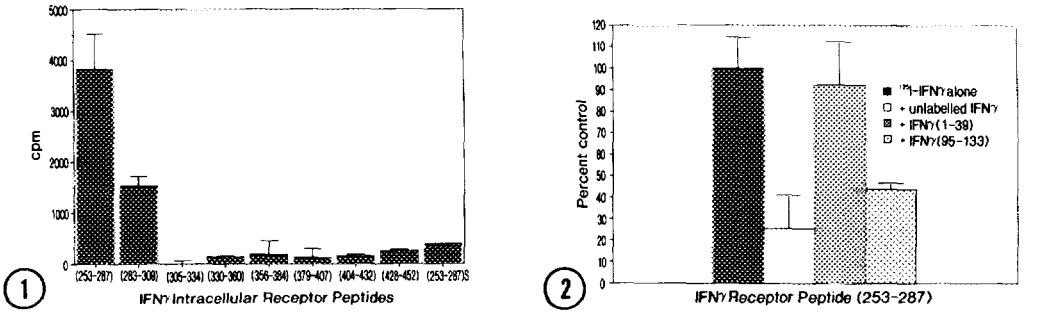

Figure 1. Binding of 5 nM ^{125}I-IFNγ to intracellular receptor peptides. ^{125}I-IFNγ, specific activity = 40-50 μCi/μg, was bound to murine IFNγ receptor intracellular peptides at a final concentration of 5 nM. Approximately one percent of the labelled IFNγ bound to peptide. Binding is expressed as a direct function of total cpm corrected for background absorbance.

Figure 2. Specific binding of ^{125}I-IFNγ to cytoplasmic receptor peptide (253-287). ^{125}I-IFNγ was used at a final concentration of 5 nM. Competitor concentrations were [IFNγ] = 1 μM, [IFNγ(1-39)] = 25 μM, [IFNγ(95-133)] = 5 μM. Competitions are described in Materials and Methods. Difference between binding of ^{125}I-IFNγ in the absence and presence of competitor IFNγ(95-133) was significant by Student's t-test, $p<0.035$. Binding is expressed as in Fig. 1.

its N-terminal and C-terminal peptides, IFNγ(1-39) and IFNγ(95-133), were all able to significantly block the binding of ^{125}I-IFNγ to the soluble form of the receptor.

Binding of IFNγ(95-133). The IFNγ C-terminal peptide, IFNγ(95-133), also bound exclusively to receptor peptide (253-287) (Figure 3). This binding was most effectively blocked by IFNγ and by IFNγ (95-133) (Figure 4). N-terminal peptide IFNγ (1-39) was

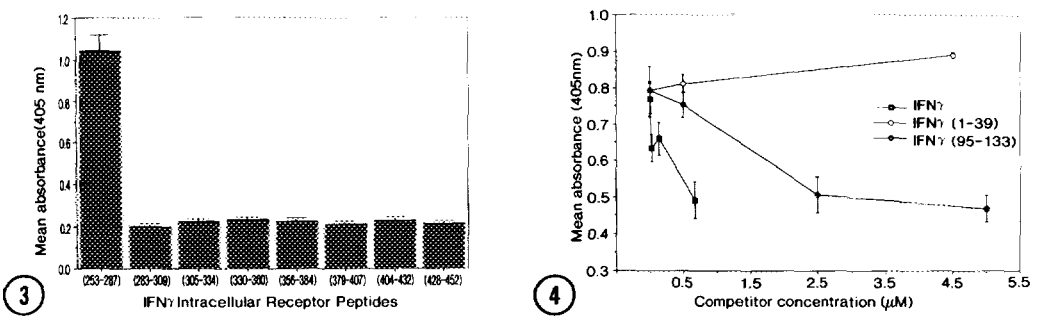

Figure 3. Binding of IFNγ(95-133) to intracellular receptor peptides. Biotinylated IFNγ(95-133) at a final concentration of 5 nM was bound to intracellular receptor peptides. Amount of peptide bound was determined colorimetrically by alkaline phosphatase activity as described in Materials and Methods. Binding is presented as the mean absorbance value at 405 nm of triplicate experiments.

Figure 4. Specific binding of IFNγ(95-133) to intracellular receptor peptide (253-287). Competitors are murine IFNγ, N-terminal peptide IFNγ(1-39), and C-terminal peptide IFNγ(95-133). Competition is described in Materials and Methods. Binding is expressed as in Fig. 3.

unable to compete for binding, providing further support that residues 253-287 of the cytoplasmic domain of the receptor indeed contain the binding site for the C-terminus of IFNγ.

Consistent with the cytoplasmic binding data, IFNγ(95-133) did not bind significantly to a truncated form of the receptor which contains only the extracellular domain or to the peptide (95-120), which binds IFNγ and IFNγ(1-39) (data not shown). As previously shown, IFNγ did bind to the truncated form of the receptor (data not shown, 11). These results further support the specificity of binding of the C-terminal peptide to the cytoplasmic domain of the IFNγ receptor.

Binding to Fixed-Permeabilized Cells and Inhibition by Specific Ab. Finally we examined the ability of IFNγ (95-133) to bind to murine L929 fibroblasts that had been both fixed and permeabilized. As shown in Table 2, IFNγ (95-133) binds to fixed/permeabilized L cells but not to untreated cells, indicating that access to the cytoplasmic portion of the receptor is necessary for binding of this ligand. Anti-peptide antiserum raised against receptor peptide (253-287) was able to abrogate binding of IFNγ (95-133) to the fixed/permeable L cells, while preimmune serum and antiserum specific for the extracellular ligand binding domain did not affect this binding (Table 2).

DISCUSSION

Studies utilizing human IFNγ receptor deletion mutants expressed in murine cells have indicated a critical role for the cytoplasmic domain of the receptor in the biological response to IFNγ. Deletion of the membrane proximal 48 residues of the cytoplasmic domain resulted in a receptor with loss of the ability to internalize and degrade IFNγ (13). This region is homologous to the murine sequence to which IFNγ and its C-terminal

Table 2. IFNγ(95-133) binds to fixed/permeabilized L cells and can be specifically blocked by anti-(253-287) antisera

Cells	Mean Fluorescence ± SD	% of Cells Stained
Untreated cells	15.1 ± 0.5	---
Fixed/Permeabilized cells	21.2 ± 0.7	---
Untreated + IFNγ(95-133)	25.2 ± 1.9	28.8 ± 3.6
Fix./Perm. + IFNγ(95-133)	34.6 ± 1.4*	52.0 ± 1.6
Fix./Perm. + IFNγ(95-133) + Anti-(253-287)	25.9 ± 0.8**	28.2 ± 1.4
Fix./Perm. + IFNγ(95-133) + Preimmune Serum	36.5 ± 3.0	---
Fix./Perm. + IFNγ(95-133) + Anti-(95-120)	33.7 ± 1.6	50.7 ± 0.8

* Difference between Untreated + IFNγ(95-133) and Fix./Perm. + IFNγ(95-133) significant by $p<0.0198$.
** Difference between Fix./Perm. + IFNγ(95-133) and Fix./Perm + IFNγ(95-133) + Anti-(253-287) significant by $p<0.0102$.
N.B. p-values were determined using Student's t-test.

peptide bind. The fact that the membrane proximal regions of the cytoplasmic domains of the murine and human IFNγ receptors share approximately 88% amino acid homology, combined with the observation that human ligand binding can be reconstituted by chimeric human/murine receptors (14), may explain previous reports that internalized IFNγ is species non-specific in its biological activity (15-17). How this relates to the requirement for a species specific cofactor is uncertain (18). Thus, the N-terminal region of IFNγ may be involved in species specific recognition and binding, while the C-terminal region may be involved in binding of internalized IFNγ to the cytoplasmic receptor domain. This binding, in turn, may be critical for initiation of events associated with IFNγ-induced kinase activity.

Finally, there is the potential that this phenomenon of a cytoplasmic ligand binding domain may not be restricted to the IFNγ receptor. The IFNγ receptor is a member of the cytokine receptor superfamily (19). Members of this superfamily, including the IL-6 receptor and the IL-2 receptor β chain have been reported to have regions of their cytoplasmic domains proximal to the membrane which are essential for ligand internalization and biologic response (20). Other members of this family, namely the erythropoietin receptor (EPOR) and the human growth hormone receptor (GHR), have been shown to directly associate with the protein tyrosine kinase JAK2 (21, 22). Interestingly, this direct association is believed to occur at the membrane proximal region of the cytoplasmic domains of these receptors, the same region to which the C-terminus of IFNγ binds. JAK2 has also recently been shown to be an integral element of the IFNγ signalling pathway (23). If this kinase associates with the IFNγ receptor as it does with the EPOR and the GHR, then direct displacement of JAK2 by first internalization and then binding of IFNγ to its cytoplasmic binding site may be a key event in initiating the signal transduction cascade.

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