Signaling by Covalent Heterodimers of Interferon-γ

EVIDENCE FOR ONE-SIDED SIGNALING IN THE ACTIVE TETRAMERIC RECEPTOR COMPLEX*

Received for publication, November 30, 1999, and in revised form, May 11, 2000
Published, JBC Papers in Press, May 15, 2000, DOI 10.1074/jbc.M909607199

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Interferon-γ (IFN-γ) and its receptor complex are dimeric and bilaterally symmetric. We created mutants of IFN-γ that bind only one IFN-γR1 chain per dimer molecule (called a monovalent IFN-γ) to see if the interaction of IFN-γ with one-half of the receptor complex is sufficient for bioactivity. Mutating a receptor-binding sequence in either AB loop of a covalent dimer of IFN-γ produced two monovalent IFN-γ, γ”γ” and γ-γ”, which cross-link to only a single soluble IFN-γR1 molecule in solution and on the cell surface. Monovalent IFN-γ competes fully with wild type IFN-γ for binding to U937 cells but only at a greater than 100-fold higher concentration than wild type IFN-γ. Monovalent IFN-γ had antiviral activity and antiproliferative activity, and it induced major histocompatibility complex class I and class II (HLA-DR) expression. In contrast, the maximal levels of activated Stat1α produced by monovalent IFN-γ after 15 min were never more than half of those produced by either wild type or covalent IFN-γ in human cell lines. These data indicate that while monovalent IFN-γ activates only one-half of a four-chain receptor complex, this is sufficient for Stat1α activation, major histocompatibility complex class I surface antigen induction, and antiviral and antiproliferative activities. Thus, while interaction with both halves of the receptor complex is required for high affinity binding of IFN-γ and efficient signal transduction, interaction with only one-half of the receptor complex is sufficient to initiate signal transduction.

IFN-γ” is a bilaterally symmetric noncovalent dimer that binds two primary ligand-binding receptor chains (IFN-γR1) in structurally equivalent positions (1, 2). We define IFN-γ as a divalent ligand because of this property. According to the current model of IFN-γ signal transduction, after ligand binding and receptor complex activation (Fig. 1), the IFN-γ receptor complex is composed of one IFN-γ dimer, two IFN-γR1 chains, two IFN-γR2 chains, two Jak1 molecules, and two Jak2 molecules (3–8). In this report, we address whether both halves of the receptor complex must be activated for signaling and whether a functional signaling receptor complex can be formed with only two receptor chains (one IFN-γR1 and one IFN-γR2 chain) instead of four chains. In order to address these questions, we designed and assayed a dimeric IFN-γ molecule that can bind only one ligand binding receptor chain (a monovalent IFN-γ).

A tandem covalently linked dimer of IFN-γ (9), designated γ-γ”, is an ideal ligand for studying the importance of ligand divalency for receptor activation because one of two receptor binding sites can be selectively eliminated to create a monovalent IFN-γ. The γ-γ” was synthesized by linking two DNA sequences encoding monomers of wild type IFN-γ, a noncovalent IFN-γ dimer designated γ-γ”, with DNA encoding a linker region from the IgA1 molecule (9). This γ-γ” had similar chromatographic properties as γ-γ and was conformationally similar to γ-γ as judged by 1H NMR (9). In binding competition studies, γ-γ” effectively competed with radiolabeled γ-γ for receptors on U937 cells, and its specific antiviral activity was 50–65% that of γ-γ (9). With the two IFN-γ monomers of γ-γ” fused, it was possible to mutate each monomer segment separately to eliminate either one of the two receptor binding sites of γ-γ”. It was previously demonstrated that mutagenesis of residues 20–25 of γ-γ” within the AB loop (1, 2) dramatically reduced the ability of γ-γ” to bind to its receptor as judged by competition studies on U937 cells (10). These residues of the AB loop are part of a 310 helix that contacts IFN-γR1 directly (2). The 1H NMR spectrum of one such mutant, IFN-γ/A23E,D24E,N25K (IFN-γ/E3K, also designated γ””-γ”), was nearly identical to that of γ-γ, demonstrating that its overall structure is retained; however, it possessed less than 0.01% of the ability of γ-γ” to compete for receptor binding to U937 cells (10). Consistent with its weak binding, this mutant possessed undetectable antiviral activity and stimulated HLA-DRα promoter activity only at over 100 nM protein (10). Thus, this mutation effectively eliminates the ability of IFN-γ” to bind to and activate its cellular receptor. Accordingly, we targeted these residues to inactivate each of the two receptor binding sites of γ-γ” to create two monovalent γ-γ molecules. This report describes how these monovalent mutants of γ-γ” were used to show that interaction of IFN-γ” with one-half of the receptor complex is sufficient to initiate signal transduction.

* This study was supported in part by NCI, National Institutes of Health (NIH), Grant ROI-CA46465 (to S. P.); NIAID, NIH, Grants ROI AI36450, ROI AI43369, and 2T32AI07403 (to S. P.); American Heart Association Grant 9730247N; New Jersey Commission on Cancer Research grants (to S. K.); and the New Jersey Commission on Cancer Research grants (to S. K.); and its receptor complex are di-
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Fig. 1. Model of receptor complex activation. IFN-γ assembles a receptor complex of two IFN-γ-R1 chains, two IFN-γ-R2 chains, two Jak1 molecules, two Jak2 molecules, and most likely yet unidentified components. This figure is taken from Kotenko et al. (4) with permission.

MATERIALS AND METHODS

Tissue Culture Media and Reagents—No antibiotics were used during propagation of cells. HEp-2 cells were grown in minimal essential medium supplemented with 10% (v/v) fetal bovine serum. HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. U937 cells were grown in RPMI medium supplemented with 1% fetal bovine serum in roller bottles at a density less than 1,000,000 cells/ml at 37 °C, 5% CO2, 99% relative humidity. To supplement with 1% fetal bovine serum in roller bottles at a density at least 2 days (1.3 × 10⁶ cells/well) prior to induction for 15 min with IFN-γ. Ligand was diluted to the working concentration at 30 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Twenty seconds prior to stop time, medium was removed, and cells were washed with 2 ml of PBS held at the same temperature. PBS was immediately removed, and at stop time (15 min), cells were scraped directly into 100 μl of ice cold lysis buffer (0.5% Brij 96, 0.03 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium vanadate, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 150 mM NaCl). After a 30-min incubation on ice, 2.5 μl of lysate was added to a mixture of 0.2 mg/ml poly(dI-dC), 1% Ficoll, 4 mM HEPES (pH 7.4), 30 mM NaCl, 1 μg/ml of an oligonucleotide encoding the gamma-activating sequence (GAS) element from the IFN-γ promoter. This oligonucleotide was prepared from a self-anneling oligomer primer that was labeled with [α-32P]dCTP by filling in the four nucleotide overhangs with the Klenow fragment of DNA polymerase I (15, 16) and has the final nucleotide sequence 5′-GATCAATTTCCCCGAAATCATG-3′ with the Stat1s consensus binding site. After incubation for 20 min at room temperature, samples were loaded onto a 5% native polyacrylamide gel and resolved by electrophoresis for 4 h at 450 V. Immediately afterward, the gel was dried under vacuum for 60 min at 80 °C and autoradiographed, or its radioactivity was quantitated with a phosphor imager. The assay was performed as described (18). Briefly, cells were grown to 10–20% confluence when induced with IFN-γ. Cells were then grown for 3 days in the continuous presence of ligand. Afterward, the cells (approximately 1 million) were washed with PBS, harvested with PBS supplemented with trypsin and 1 mM EDTA, and treated with 50 μl of conditioned medium from a hybridoma secreting W6/32 murine monoclonal antibody (about 1 μg/ml) together with an epitope specific for the HLA-B7 class I antigen (19). After a wash with PBS, fluorescein isothiocyanate-conjugated secondary anti-mouse antibody was added to the cells, and cells were analyzed by flow cytometry with a Coulter II flow cytometer.

HLA-DRα Reporter Gene Bioassay—The assay was performed as described previously (26). Briefly, the promoter region of the HLA-DRα gene (the HLA-DRA gene) was linked to the coding region of the growth
three times with PBS containing 5% Tween 20 (v/v). The $^{125}$I-INF-$\gamma$ bound was removed with 50 $\mu$L of 0.2 M NaOH containing 1% SDS. Radioactivity was counted with an LKB model 1272 $\gamma$-counter. Scatchard analyses were performed as described (21).

**Cross-linking Procedure—**Cross-linking to soluble receptors was carried out as described previously (13). Briefly, 16 $\mu$L (12.5 $\mu$g) recombinant soluble Hu-INF-$\gamma$-R1EC produced from COS cells was incubated with 4 $\mu$L (2.5 $\mu$g) INF-$\gamma$ in 50 $\mu$L of PBS, and, after a 30-min incubation period, cross-linking was initiated by adding 5 $\mu$L of 10 mM bis(sulfosuccinimidyl)suberate. After 30-min cross-linking, the reaction was stopped by the addition of 10 $\mu$L of 1 M Tris-HCl, pH 7.0. Reaction products were analyzed by SDS-PAGE and visualized by Coomassie Blue staining.

Cross-linking to cell surface receptors was performed as described (3) with slight modification. Briefly, $^{125}$I-labeled INF-$\gamma$ species (2 $\mu$m $\gamma$-$\gamma$ or 20 $\mu$m $\gamma$-$\gamma$) was added to 1 $\times$ 10$^6$ U937 cells in 200 $\mu$L of PBS containing 0.1% bovine serum albumin, followed by the addition of a noncleavable cross-linker EDC/sulfosuccinimidyl (10 mM each; EDC added immediately before sulfosuccinimidyl) and further incubation for 60 min at ambient temperature. The reaction was stopped by the addition of 0.1 volume of 1 M glycine. Cells were washed once with PBS and then lysed as described (3). The whole cell lysate was analyzed by SDS-polyacrylamide gel electrophoresis, and the cross-linked products were visualized by autoradiography.

**Antiviral Assays—**Antiviral assays were performed essentially as previously reported (22). HeLa cells (35,000 per well) were plated in 96-well microtiter plates containing 12 2-fold serial dilutions of IFN-$\gamma$. After 24 h, vesicular stomatitis virus was added at 5000 plaque-forming units/well. When cells in viral control wells containing no interferon were lysed (usually about 48 h), plates were drained and stained with crystal violet to visualize live cells.

**RESULTS**

**Stoichiometry of Ligand Receptor Interactions—**The stoichiometry of the covalent dimer of IFN-$\gamma$-$\gamma$ with the soluble INF-$\gamma$-R1 extracellular domain (INF-$\gamma$-R1EC) was examined by gel filtration to ensure that the $\gamma$-$\gamma$ could bind two soluble INF-$\gamma$-R1 (INF-$\gamma$-R1EC) molecules. It was previously demonstrated that wild type INF-$\gamma$-$\gamma$ (33–34 kDa) can bind to two INF-$\gamma$-R1EC (80 kDa) or two cell surface receptors simultaneously (3, 12, 13). Thus, a soluble ternary complex would have a molecular mass of 114 kDa, and a binary complex would have a molecular mass of 74 kDa. As shown in Fig. 3, both $\gamma$-$\gamma$ and $\gamma$ form receptor complexes greater than 100 and 130 kDa apparent molecular mass, respectively, independent of the ratio of ligand to receptor. Our results with $\gamma$-$\gamma$ agree well with the gel filtration data reported for $\gamma$-$\gamma$ (3) and were repeated here as a positive control. Western blotting confirmed that fractions containing the higher molecular weight complex were composed of both ligand and receptor, while fractions containing the lower molecular weight complex were composed almost entirely of the component in excess, either ligand or receptor (data not shown). Small traces of the high molecular weight complex are present in the fractions containing the lower molecular weight component because the two peaks were not completely resolved. The $\gamma$-$\gamma$-INF-$\gamma$-R1EC complex may have a lower apparent molecular weight because either its complex may be less stable than the $\gamma$-$\gamma$-INF-$\gamma$-R1EC complex during gel filtration or the hinge region of $\gamma$-$\gamma$ may alter the structure of the ternary complex.

Although the ($\gamma$-$\gamma$)-INF-$\gamma$-R1EC and ($\gamma$-$\gamma$)-INF-$\gamma$-R1EC complexes were consistently above 100 and 130 kDa, respectively, variability in the physical structures or stability of proteins and protein complexes make accurate molecular weight and stoichiometric determinations from a size exclusion column ambiguous. To confirm the stoichiometry of each ligand-receptor complex, we cross-linked the ligand-soluble receptor complexes, analyzed the products by SDS-polyacrylamide gel electrophoresis, and estimated their aggregate apparent molecular weights. As shown in Fig. 4A, $\gamma$-$\gamma$ formed complexes of 70–75 and 105–115 kDa (lane 7). This represented complexes L$_2$R and
**Fig. 3. Gel filtration studies of complexes of γ-γ and γ-γ to soluble IFN-γR1**

Between 0.5 and 5 μg of ligand and between 5 and 40 μg of IFN-γ-R1 were mixed and incubated at 4 °C for 30 min before chromatography. All chromatograms are drawn to scale horizontally and normalized vertically by maximum peak height. **Top panel**, elution profiles for γ-γ, γ-γ', and IFN-γ-R1 are shown. Molecular weight standards for column calibration are indicated by the arrows designating the position of elution of the corresponding molecular weight markers. **Middle panel**, complexes of γ-γ with IFN-γ-R1. The numbers represent the input molar ratio of γ-γ to soluble IFN-γ-R1. Complexes formed at ligand: receptor input molar ratios of 0.23 and 3.51 (gray and black, respectively) are shown. **Bottom panel**, complexes of γ-γ with IFN-γ-R1. Input ligand:receptor molar ratios of 0.21 and 2.47 (gray and black, respectively) are shown.
If IFN-γ must be divalent to bind to the cell surface with high affinity, then the residual competitive ability of γm-γ and γ-γm could result from a reconstitution of a divalent wild type IFN-γ ligand from fragments resulting from degradation of the monovalent IFN-γ. Alternatively, this reconstituted divalent IFN-γ could be produced by intermolecular association of two intact covalent heterodimers to produce a covalent tetramer acting as a divalent IFN-γ. To examine the possibility that a reconstituted divalent IFN-γ is the active species in γ-γm preparations, binding studies of γ-γm and γm-γ were performed with IFN-γR1EC-coated plates and were analyzed by the method of Scatchard (21). Fig. 6 shows the results of the Scatchard plots of the two ligands. The divalent γ-γ possesses two binding affinities for the IFN-γR1EC immobilized to the microtiter plates. Five to ten percent of the binding sites on the plates have an affinity for γ-γ of 150 pM, identical to the affinity found for γ-γ on U937 cells by Rashidbaigi et al. (24). We believe that this affinity represents the true affinity of γ-γ for cellular receptors present on the U937 cell surface, because in the cellular competition assays all of the prebound radiolabeled γ-γ was displaced with less than 1 nM γ-γ (Fig. 5). The remaining binding sites on the plate have an affinity for γ-γ of about 10 nM. γ-γm has only the 10 nM affinity for the immobilized receptors, with no trace of the high affinity site. This affinity also resembles that observed in the cellular competition assay. Because no high affinity binding was observed with γ-γm, we conclude that there is no reconstituted γ-γm divalent ligand in the γ-γm preparation and that all cellular receptor binding activity is a direct result of binding of the intact γ-γm to the receptors. Consistent with the observation that high affinity binding required ligand divalency, both γ-γ and γ-γm possess the same low affinity constant for immobilized IFN-γR1EC. The origin of the nanomolar affinity binding for γ-γ may be related to the lack of specific orientation of IFN-γR1EC molecules on the plate. Because the recep-
tors on the surface of the microtiter plate are probably immobilized randomly, only a small fraction of the receptors may be oriented to allow divalent ligand binding to two IFN-γR1EC molecules. Therefore, even with divalent ligand preparations, the predominant binding may occur to single immobilized IFN-γR1EC molecules. Thus, the 10 nM affinity of γ-γ probably represents the affinity of γ-γ for a single IFN-γR1EC.

The above results demonstrate that the heterodimeric γ-γ (γ-γm and γm-γ) are monovalent ligands with severely reduced affinity for cellular receptors. No evidence for divalent IFN-γ present in the γm-γ and γ-γm preparations was observed.

Antiviral, MHC Class I Antigen Induction, HLA-DRA, and Stat1α Assays—To test the hypothesis that a single receptor activation event is sufficient to activate IFN-γ signal transduction, we employed several bioassays. Both divalent IFN-γ (γ-γ and γ-γm) and monovalent IFN-γ (γm-γ and γ-γm) possessed antiviral activity (Fig. 7). The cytopathic effect end point (ED50) with HeLa cells was 3 pM (1 unit/ml) for γ-γ and 5 pM for γ-γm, as previously reported by Lunn et al. (9) (Table I). The ED50 was 34 nM for γ-γm and 390 pM for γ-γm. At much higher concentrations of IFN-γ, both monovalent as well as divalent IFN-γ began to exhibit antiproliferative effects on HeLa cells, as seen in the leftmost wells in Fig. 7. Analysis of the slowly proliferating cells by light microscopy confirmed that all cells appeared uninfected and alive.

MHC class I surface antigen expression was induced by 3 days of treatment of HEP-2 cells by monovalent and divalent IFN-γ (Fig. 8). Upon induction by 300 nM γ-γm and 90 nM γ-γ, the MHC class I surface antigen expression was equivalent to that induced by 3 nM γ-γ or 3 nM γ-γm. We also assayed the ability of the four IFN-γs to activate the HLA-DRα promoter controlling growth hormone (GH) expression in HeLa cells. Both monovalent and divalent IFN-γ were able to maximally induce GH secretion in response to IFN-γ treatment (Fig. 9). Consistent with previous results, much higher concentrations of monovalent IFN-γ (1.5 nM γm-γ and 40 pM γ-γm) were necessary for half-maximal increases in GH secretion than required for divalent IFN-γ (0.3 pM γ-γ and 3.5 pM γ-γm). As a control, the IFN-γ mutant that contains the EEK mutation (γm-γm) on both chains was simultaneously assayed. It possessed much less potency than either monovalent ligand, with an ED50 of 100 nM for HLA-DR-driven GH secretion (Fig. 9) in agreement with previous results (10).

Stat1α activation is an early event of IFN-γ signal transduc-
tion necessary for both antiviral activity and induction of various genes (25–27). The abilities of the IFN-γ variants to activate Stat1a in human HeLa cells after 15 min are shown in Fig. 10. The results are similar with other human cell lines (Table I). Stat1a is activated by both monovalent and divalent IFN-γ, and the extent of activation increases with increasing IFN-γ concentration. Approximately 6 pM γ-γ yields a half-maximal activation level of Stat1a in HeLa cells. Although γ-γ activates as much Stat1a as γ-γ at its optimal levels, the EC50 of 300 pM for γ-γ in this assay was 50-fold higher. Monovalent IFN-γ activated Stat1a only at much higher concentrations. Half-maximal Stat1a activation was observed at 80 nM γ-γ.

Fig. 7. Antiviral assays of the IFN-γs on HeLa cells. Cells were plated in the presence of serial 2-fold dilutions of various IFN-γs. After 24 h, vesicular stomatitis virus was added. After 48 h, plates were drained and stained with crystal violet to visualize viable cells. The first well of each row contained (from top to bottom), 1.5 nM (500 units/ml) γ-γ, 3 nM γ-γ, 1200 nM γ-γ, and 120 nM γ-γ in a volume of 0.2 ml. The bottom row represents six positive cell controls (neither vesicular stomatitis virus nor IFN-γ) on the left and six virus controls (VSV but no IFN-γ) on the right. Details of the assays are described under “Materials and Methods.”

TABLE I

Activities of the various Hu-IFN-γs

Each IFN-γ was assayed for various biological activities. Concentrations in pM required to elicit half-maximal response for the various activities are tabulated along with the cell lines used for each assay. For Stat1a data, the first number represents the EC50 of the IFN-γ, while the number in parentheses represents the maximal level of phosphorylated Stat1a in 15 min relative to that of γ-γ, arbitrarily set as 100. MHC class I data are presented as a pair. The first number is the picomolar concentration of IFN-γ required to induce maximal MHC class I antigen expression. The second number is the ratio of fluorescence of cells binding W6/32:fluorescein isothiocyanate-conjugated anti-murine IgG complexes after IFN-γ treatment relative to fluorescence of untreated cells.

| Activity       | Cell line | γ-γ | γ-γ | γ-γm | γ-γm |
|----------------|-----------|-----|-----|------|------|
| Competition (IC50) | U937     | 20  | 30  | 9000 | 3500 |
| anti-VSV (EC50)  | HeLa     | 3   | 5   | 36,000 | 392  |
| Antigrowth      | HeLa     | 400 | 400 | >1,200,000 | 60,000 |
| HLA-DRa         | HeLa     | 0.3 | 3.5 | 1500  | 40   |
| MHC Class I     | HEP-2    | (2,900, 2.7) | (2,900, 2.9) | (284,000, 3.1) | (88,000, 3.1) |
| Stat1a          | HEP-2    | 3.01 (100) | 1234 (127) | 183,600 (19) | 3845 (62) |
| Stat1a          | HeLa     | 6.618 (100) | 323 (112) | 82,750 (25) | 7268 (62) |
| Stat1a          | COS-1    | 2.53 (100) | 256 (111) | 64,920 (20) | 2821 (55) |
| Stat1a          | HL-60    | 1.78 (100) | 59.10 (83) | 2392 (64) | 737 (45) |
| Stat1a          | U937     | 1.78 (100) | 35.8 (76) | 9785 (37) | 1149 (46) |

Fig. 8. MHC class I surface antigen induction in HEP-2 cells by the IFN-γs. The MHC class I antigen assays were performed as described under “Materials and Methods.” Unfilled histograms represent base-line expression of MHC class I antigen expression on untreated cells. Filled histograms represent the MHC class I antigen expression on untreated cells. The data represent analysis of 10,000 cells by flow cytometry.
and 7 nM \( \gamma-\gamma^m \). Interestingly, the maximal levels of Stat1\( \alpha \) activated by \( \gamma-\gamma^m \) in HeLa cells were only about 50% of that activated by \( \gamma-\gamma \) or by \( \gamma-\gamma \). In other human cell lines tested, the maximal levels of Stat1\( \alpha \) activated by \( \gamma^m-\gamma \) or \( \gamma-\gamma^m \) were never more than about half that of \( \gamma-\gamma \) or \( \gamma-\gamma \) (Table I). These results are consistent with the hypothesis that the Jak/STAT pathway can be activated by monovalent IFN-\( \gamma \) binding to and activating one-half of the receptor complex. Thus, it would seem that while only a single side of the IFN-\( \gamma \) receptor complex needs to be activated to elicit IFN-\( \gamma \) bioactivity, ligand divalency is required for a maximal Stat1\( \alpha \) response as well as for high affinity receptor binding.

**DISCUSSION**

Our results show that \( \gamma-\gamma \), like \( \gamma-\gamma \), was capable of binding two soluble receptors as demonstrated by gel filtration chromatography and by covalent cross-linking to two soluble receptors and to two cell surface IFN-\( \gamma \)R1 chains (Figs. 3 and 4, A and B). The Ala-Asp-Asn sequence in the AB loop at the NH\(_2\) terminus of \( \gamma-\gamma \) resides in the portion of \( \gamma-\gamma \) that closely resembles either half of \( \gamma-\gamma \). Mutation of this sequence to create \( \gamma^m-\gamma \) results in a molecule that can only signal with the half that contains AB loop near the IgA\(_2\) hinge (Fig. 11). Mutation of the analogous sequence in the COOH-terminal half of the primary structure of \( \gamma-\gamma \) to create \( \gamma-\gamma^m \) eliminates receptor binding in the hinged region. \( \gamma^m-\gamma \) can bind to receptors only with the half that resembles either half of \( \gamma-\gamma \) (Fig. 11). Each monovalent IFN-\( \gamma \) can cross-link to only one soluble receptor, and \( \gamma-\gamma^m \) can cross-link efficiently to only one cell surface IFN-\( \gamma \)R1 (Fig. 4, A and B), demonstrating that this mutation in \( \gamma-\gamma \) created monovalent IFN-\( \gamma \). The hinge region, perhaps by restricting the optimal conformations of the termini of IFN-\( \gamma \), impairs both the binding and the biological activity of IFN-\( \gamma \), as seen in comparing the binding and activity of \( \gamma-\gamma \) with those of \( \gamma^m-\gamma \) and by comparing the binding and activity of \( \gamma^m-\gamma \) with those of \( \gamma-\gamma^m \) (Table I). The relative placement of the mutations in the AB loop with respect to the hinge appears to distinguish the \( \gamma^m-\gamma \) and \( \gamma-\gamma^m \) variants. Both \( \gamma^m-\gamma \) and \( \gamma-\gamma \) (whose receptor binding site is close to the hinge) are less efficient at competition and have less biological activity than do \( \gamma-\gamma^m \) and \( \gamma-\gamma \), respectively (Table I).

The monovalent IFN-\( \gamma \)-haad considerably decreased affinity for the cell surface receptor as shown in competition studies and solid-phase binding assays (Figs. 5 and 6). The nanomolar affinity of \( \gamma-\gamma^m \) and \( \gamma^m-\gamma \) does not appear to arise from trace...
contaminants of divalent IFN-γ because 1) divalent IFN-γ had two saturable specific binding sites in the solid-phase receptor binding assay, while γ-γ'' had only a single binding site (Fig. 6); 2) divalent IFN-γ formed a 105–115-kDa complex when cross-linked with the soluble IFN-γR1KC chain in addition to the 70–75-kDa complex, while γ-γ'' and γ''-γ formed only the 70–75-kDa complex (Fig. 4A); and 3) monovalent IFN-γ never activated as much Stat1α in 15 min as did γ-γ or γ-γ in any human cell line tested (Fig. 10A, Table I). If any activity in the γ''-γ or γ-γ'' preparations resulted from the presence of a divalent IFN-γ contamination, the maximum Stat1α activation ultimately would be the same for γ''-γ and γ-γ'' as for γ-γ or γ-γ. However, this was not seen (Fig. 10, Table I). All of these data led us to conclude that there is no detectable trace of divalent ligand in the monovalent IFN-γ preparations and that all residual activity from the monovalent ligand preparations arose from the binding of monovalent ligands to low affinity sites on the cell surface. Both γ-γ'' and γ-γ have the same 10 nM affinity for the immobilized IFN-γR1KC (Fig. 6). If the 10 nM affinity seen with γ-γ represents an intermediate binary complex before formation of the ternary γ-γ(IFN-γR1) complex (Figs. 3 and 4A), divalency of IFN-γ is apparently required for high affinity binding. Alternatively, the increased affinity with divalent ligand binding may be mediated by avidity. Avidity is a term often used to describe an increase in apparent affinity of multivalent antibodies relative to that of monovalent Fab fragments due to multiple interactions, each having identical affinity with an antigen. Significantly, the gel filtration experiments show that although a ternary complex between γ-γ or γ-γ and IFN-γR1KC is spontaneously formed, even at low IFN-γR1KC:ligand ratios, the apparent molecular weight of the ligand-receptor complex gradually increases as more receptor is titrated into the reaction. This is consistent with a rapid binary:ternary equilibrium between γ-γ or γ''-γ and soluble IFN-γR1KC. Soluble IFN-γR1KC can displace γ-γ from the IFN-γ receptor complex on the cell surface and inhibit signal transduction, indicating that this equilibrium does indeed occur on the cell surface as well as in solution (3). Since a single receptor activation event is sufficient to trigger IFN-γ signaling, the use of soluble IFN-γ receptors as IFN-γ signaling antagonists may not be as effective as growth hormone (28), because IFN-γ bound to one soluble IFN-γR1 and one cell surface IFN-γR1 would signal according to our results.

Remarkably, monovalent IFN-γ has full biological activity. This contrasts with results obtained with growth hormone where mutation of the second receptor binding site creates an antagonist of the receptor complex (28). Randal and Kossiakoff (29) noted that their monovalent IFN-γ was also active in signal transduction but provided no supporting biological data. Our monovalent IFN-γ exhibited antiviral and antiproliferative activity (Table I, Fig. 7). It activated the HLA-DRα promoter and induced MHC class I surface antigen expression (Table I, Figs. 8 and 9). In accord with these activities, our
signaling network is likely to exist in which one of the receptor's chains is defective and that one-sided signaling is sufficient for IFN-γ signal transduction. A monomeric fragment of human IFN-γ, consisting of amino acids 95–134, was reported to bind to amino acids 253–287 within the cytoplasmic domain of human IFN-γR1 with a dissociation constant of 37 nM in vitro (30). This monomeric peptide expressed in cells induced MHC class II surface antigen expression and conferred antiviral-antigenostis virus protection without species specificity (31). These studies also infer that a single receptor activation event can initiate IFN-γ signaling; however, the stoichiometry of peptide to receptor complex was not reported. Thus it cannot be excluded that two peptide molecules per receptor complex may be required to initiate signaling.

The concentrations of γ-γ and γ-γ required for binding competition, antiviral, and antiproliferative activities differ by only a factor of 2 (Fig. 7, Table I), suggesting a minimal influence from the IgA1 linker segment on these activities. In contrast, 10–50-fold higher levels of γ-γ than γ-γ are required for full activation of the Jak/STAT pathway or activation of the HLA-DRα promoter. Moreover, both γ-γ and γ-γ induce anti-IFN activity at much lower concentrations than those needed to observe significant levels of Stat1 activation, a phenomenon we have observed with several type I interferons2 (Figs. 8 and 10, Table I). This surprising result supports the hypothesis that induction of antiviral and antiproliferative activity could involve many different components in addition to Stat1α activation, which is necessary but not sufficient to elicit these activities (26, 27, 32, 33). Alternatively, a pathway that does not require Stat1α activation may mediate some of these uncharacterized pathways. Therefore, a more complex IFN-γ signaling network is likely to exist in which only a fraction of the components have yet been identified. Our studies provide a basis to begin to elucidate these pathways and their components.

In summary, monovalent IFN-γ that only activates one receptor chain is active in signal transduction, and activation of a single side of the IFN-γ receptor complex is sufficient to yield full IFN-γ bioactivity. However, interaction of ligand with both sides of the complex is required for high affinity activity of IFN-γ and maximal, efficient activation of the IFN-γ receptor complex.

Acknowledgments—We thank Michael Newlon and Jerome Langer for discussions and critical review of this manuscript and Eleanor Kells for assistance in the preparation of this manuscript.

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C. D. Krause, J. Cook, and S. Pestka, unpublished observations.