Alignment of Disulfide Bonds of the Extracellular Domain of the Interferon γ Receptor and Investigation of Their Role in Biological Activity

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ABSTRACT: The extracellular ligand binding domain of the human interferon γ receptor includes eight cysteine residues forming four disulfide bonds. Only the nonreduced protein binds interferon γ. We investigated the alignment of the disulfide bonds, using an enzymatically deglycosylated form of a soluble interferon γ receptor, produced in baculovirus-infected insect cells. The soluble receptor was digested with endoproteinase Glu-C and proteinase K, and the proteolytic fragments were characterized by amino acid sequence analysis and mass spectrometry. It was found that four consecutive disulfide bonds are formed between residues Cys^{60}–Cys^{68}, Cys^{105}–Cys^{150}, Cys^{178}–Cys^{183}, and Cys^{197}–Cys^{218}. We also investigated the role of the disulfide bonds in biological activity of the receptor, using site-directed mutagenesis and by exchanging the cysteine residues for serines. The mutated proteins were expressed in *Escherichia coli* and analyzed for ligand binding capacity on protein blots. The assays showed that all disulfide bonds are essential for full ligand binding capacity. Double or quadruple mutations at cysteine residues 60 and 68, and residues 178, 183, 197, and 218, respectively, resulted in complete loss of the activity, whereas double mutations at residues 105 and 150, 178 and 183, and 197 and 218, respectively, resulted in a residual activity about 1 order of magnitude lower than that of the wild type. The specific antibodies γR38 and γR99 detected conformational epitopes stabilized by disulfide bonds involving cysteine residues 60 and 68, and 178 and 183, respectively.

Interferon γ (IFNγ)[1] exerts its complex functions in the control of immunological and inflammatory responses by binding to a specific cell receptor (Trinchieri & Perrussia, 1985; Langer & Pestka, 1988; Landolfo & Garotta, 1991). The receptor for human IFNγ is a 90-kDa glycoprotein consisting of an extracellular domain, a transmembrane region, and a cytoplasmic region. It does not require glycosylation in order to bind IFNγ but loses its ligand binding capacity when treated with reducing agents (Calderon et al., 1988; Fountoulakis et al., 1989; Fischer et al., 1990; van Loon et al., 1991). We previously described that it is reactivated after removal of the reductants by dialysis and concluded that it contains at least one essential disulfide bond (Fountoulakis et al., 1989). On SDS–PAGE, the receptor shows a shift in mobility when reduced which also suggests that at least one of the disulfide bonds is essential for the compactness and folding of the protein (Fountoulakis et al., 1990). The extracellular ligand binding domain of the receptor includes eight cysteine residues all of which are oxidized, forming four disulfide bonds.

After the molecular cloning of the human IFNγ receptor (Aguet et al., 1988; Gray et al., 1989), soluble forms of the IFNγ receptor have been engineered in *Escherichia coli*, baculovirus-infected insect (Sf9) cells, and Chinese hamster ovary cells and isolated in active forms. They comprise the extracellular domain of the native protein and bind IFNγ with an affinity 2–50-fold lower than that of the cellular receptor (Fountoulakis et al., 1990; Gentz et al., 1992). Using the *E. coli*-derived soluble receptor, we found that almost the whole extracellular domain of the protein is required for full ligand binding capacity and that cysteine residues 60 and 68 form a disulfide bond (Fountoulakis et al., 1991a).

In order to facilitate a structure-based design of IFNγ antagonists, which are potential pharmaceuticals against several disorders (Garotta et al., 1989), we investigated the alignment of the disulfide bonds of the extracellular domain of the human IFNγ receptor and asked which of the cysteine residues are involved in the formation of the essential disulfide bond(s). For the study of the connectivities between the cysteine residues, we used the soluble IFNγ receptor produced in insect Sf9 cells. For the study of the role of the disulfide bonds in the activity of the receptor, we replaced the cysteines with serines by using site-directed mutagenesis, expressed the proteins in *E. coli*, and analyzed the mutated soluble IFNγ receptors for ligand binding capacity.

EXPERIMENTAL PROCEDURES

Materials. Reagents for the preparation of SDS–polyacrylamide gels and low molecular mass protein size markers were from Bio-Rad. ^{14}C-Labeled protein size markers and iodinated sheep anti-mouse Ig were from Amersham. Nitrocellulose membrane was purchased from Schleicher & Schuell. Coomassie brilliant blue R-250 was from Serva. Sequencing-grade proteolytic enzymes and *N*-glycosidase F were purchased from Boehringer Mannheim.

IFNγ. IFNγ was expressed in *E. coli* and purified from large-scale fermentations by hydrophobic interaction and size-exclusion chromatography. It was iodinated utilizing the chloramine-T method (Greenwood et al., 1963) to 2×10^5 cpm/ng of protein.

Soluble IFNγ Receptor Produced in Sf9 Cells. The protein was purified from cell culture supernatants essentially like the soluble mouse IFNγ receptor produced in the same expression system (Fountoulakis et al., 1991b). The protein comprises the extracellular domain of the native receptor

[1] Abbreviations: IFNγ, interferon γ; GRH, growth hormone; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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(residues 1–229), does not include the signal peptide sequence, and starts with Glu-Met-Gly-Thr-. It is heterogeneously glycosylated and migrates with an average apparent molecular mass of about 32 kDa on nonreducing SDS–polyacrylamide gels (Figure 1A, lane 1). After removal of the carbohydrate, it migrates at 26 kDa (Figure 1A, lane 2).

Soluble IFNγ Receptor Produced in E. coli Cells. The protein comprises the extracellular domain of the native sequence, residues 1–229. In addition, it contains the amino acids Met-Arg-Gly-Ser-Arg-Ala- at its amino-terminal end (Ser-Arg-Ala- are residues of the signal peptide) and Arg-Ser-His-His-His-His-His-His at its carboxyl-terminal end. The mutants were constructed by site-directed mutagenesis (Sayers et al., 1988) and have one or more cysteine residues substituted with serines. Both wild-type and mutant forms of the receptor were expressed in *E. coli* and purified as previously described (Stüber et al., 1990).

Antibodies. The monoclonal antibodies γR38 and γR99 were raised against the native IFNγ receptor. They detect conformational epitopes in the regions of the amino acid residues 9–116 and 53–193, respectively. Furthermore, they inhibit the ligand binding capacity of the receptor and the IFNγ-mediated antiviral activity (Garotta et al., 1990). The polyclonal antibody R3891 was raised against the recombinant soluble IFNγ receptor produced in *E. coli*.

Analytical Methods. The soluble receptors, mutated and wild type, were resolved on 12% SDS–polyacrylamide gels and revealed by staining with Coomassie blue. If not otherwise indicated, no reducing agent was present in the sample buffer. SDS–PAGE-resolved proteins were electrotransferred to nitrocellulose membranes, and the binding capacities of the proteins were analyzed by ligand blots and immunoblots, as described (Fountoulakis et al., 1989). The protein concentration was determined by amino acid analysis (Fountoulakis et al., 1992a).

Enzymatic Removal of Carbohydrate. One hundred micrograms of receptor from Sf9 cells in 500 μL of phosphate-buffered saline, pH 7.4, was incubated with 5 units of *N*-glycosidase at 37 °C for 2 days. The deglycosylation process was followed by SDS–PAGE. More than 90% of carbohydrate was usually removed. *N*-Glycosidase was separated by binding the protein mixture to a Polybuffer Exchanger column. The enzyme was recovered in the flow-through fraction. The deglycosylated receptor was eluted with 1 M NaCl, and the eluate was dialyzed against water to remove the salt.

Digestion of the IFNγ Receptor. The deglycosylated, insect cell-derived receptor, in 0.1 M ammonium bicarbonate buffer, pH 7.8, was incubated with either endoproteinase Glu-C or endoproteinase Glu-C and proteinase K (receptor:enzyme ratio 25:1, each) at 37 °C for 24 h. The reaction was stopped by the addition of phenylmethanesulfonyl fluoride to a final concentration of 1 mM.

Amino Acid Sequence Analysis. The proteolytic products, nonreduced or after reduction by addition of 20 mM dithiothreitol and heating at 37 °C for 3 h, were separated by reversed-phase HPLC developed with a linear gradient of 0–65% acetonitrile containing 0.1% trifluoroacetic acid on a Hewlett-Packard 1090A system. Selected peptide-containing fractions were subjected to amino-terminal sequence analysis on an Applied Biosystems 477A sequenator equipped with an on-line phenylthiohydantoin-amino acid analyzer (Hewick et al., 1981).

Mass Spectrometry. Fragments of interest were analyzed by electrospray mass spectrometry on an API III system, SCIEX. The proteins were dissolved in 1 M acetic acid in water/methanol (50:50, v/v), and 10 μL was injected via a Hewlett-Packard 1090A system. Mass analysis was performed in the positive ion mode. Scans between m/z 400 and 2000 in 0.2 average mass unit steps were recorded and summed up.

RESULTS

Alignment of Disulfide Bonds. The connectivities between the eight cysteine residues of the extracellular domain of the IFNγ receptor were studied by using the soluble receptor produced in Sf9 cells (Gentz et al., 1992). Since carbohydrate residues interfered with molecular mass determinations of the peptides by mass spectrometry, the enzymatically deglycosylated form of the receptor was used (Figure 1A, lane 2). The nonglycosylated, *E. coli*-derived, soluble receptor was not considered for these experiments, since this protein contained about 15% of nonnative conformational forms with wrong shuffling of disulfide bonds (Fountoulakis et al., 1990). The insect cell-derived protein did not include nonnative conformational forms as judged by ligand blots and immunoblots (Fountoulakis & Gentz, 1992).

The deglycosylated receptor obtained from recombinant Sf9 cells was subjected to single or double digestion with endoproteinase Glu-C and proteinase K. Nonreduced and reduced peptide digests were separated by reversed-phase HPLC (Figure 1B,C; only the maps of the nonreduced peptides are shown). Peptide peaks, present in the maps of the nonreduced probes and absent in the maps of the reduced probes, were likely to be involved in formation of disulfide bonds, and were, therefore, subjected to N-terminal sequence analysis, and their masses were determined by electrospray mass spectrometry. Assignment of the disulfide bonds was made by comparing the found sequences of the nonreduced peptides with the deduced sequence of the undigested receptor (Figure 2).

Digestion with endoproteinase Glu-C yielded peptide a (Figure 2) which includes Cys^{60} and Cys^{68}. The peptide had the expected mass of 4346.9 (within experimental error) which means that Cys^{60} and Cys^{68} are connected with a disulfide bond, as we have previously described (Fountoulakis et al., 1991a). The same digestion delivered peptide b comprising the sequences Ile^{166}–Glu^{201} and Val^{217}–Ser^{229} (lacking the stretch Gly^{202}–Glu^{216}) and including cysteine residues 178, 183, 197, and 218. Thus, none of these cysteine residues are involved in disulfide bond formation with residues 105 or 150.

Consecutive digestion with endoproteinase Glu-C and proteinase K gave rise to peptide d which contained Cys^{178} and Cys^{183} (several peptides similar to the shown peptide d were isolated from different digestions of the receptor and different HPLC runs). The determined masses of all these peptides agreed with the predicted ones, indicating that Cys^{178} and Cys^{183} form a disulfide bond. Peptide e yielded two sequences (Tyr^{196}–Ser^{199} and Val^{217}–Thr^{220}), showing that these sequences are connected with a disulfide bond between Cys^{197} and Cys^{218}. Two sequences were assigned to peptide c, Ala^{103}–Lys^{119} and Thr^{148}–Arg^{153} containing Cys^{105} and Cys^{150} residues, respectively. A mass for this peptide could not be determined. However, since amino acid sequence analysis and mass spectrometry data unambiguously showed the existence of three disulfide bonds between Cys^{60}–Cys^{68}, Cys^{178}–Cys^{183}, and Cys^{197}–Cys^{218}, and the receptor had no free thiol groups (Fountoulakis et al., 1991a), we concluded that Cys^{105} and Cys^{150} must be connected with a disulfide bond.

Role of Disulfide Bonds in the Activity of the IFNγ Receptor. Preparation of Mutant IFNγ Receptors. We

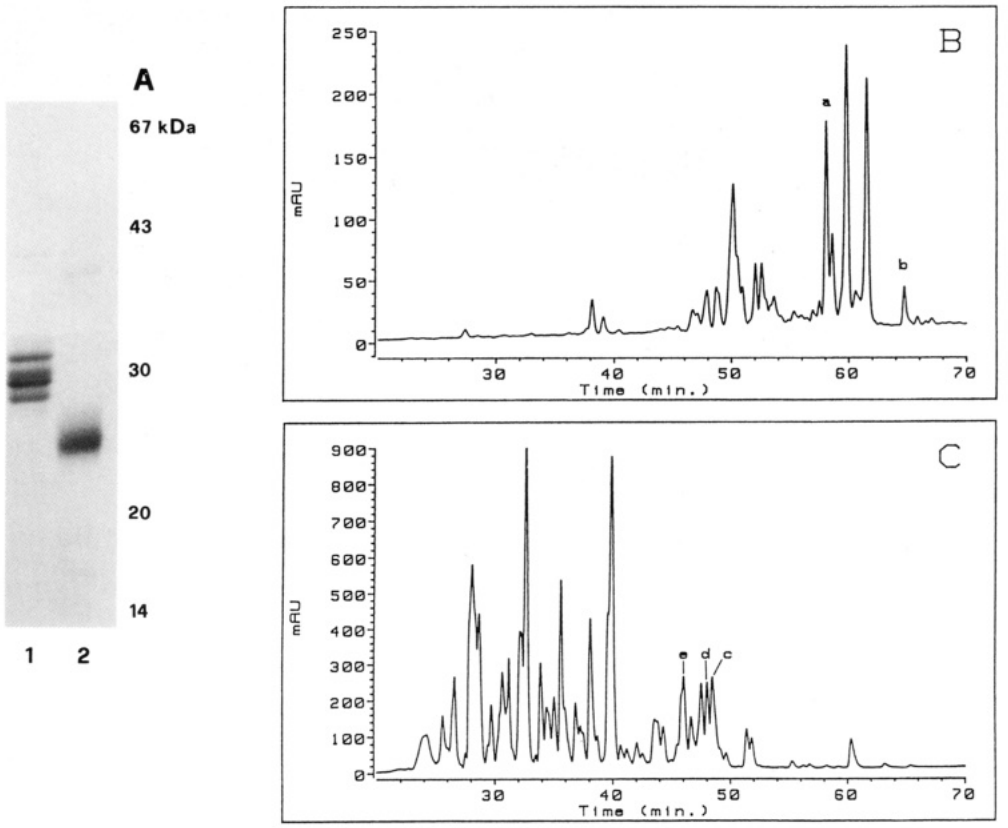


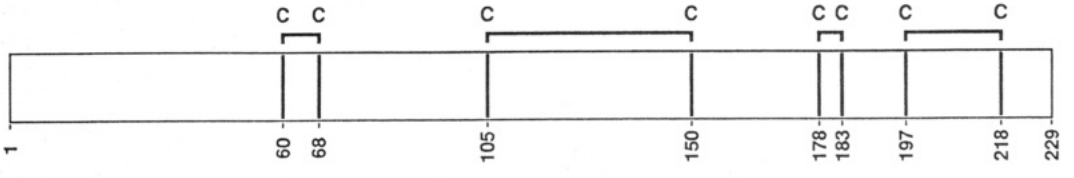
FIGURE 1: SDS–PAGE analysis of the glycosylated and deglycosylated soluble IFNγ receptor from insect cells (A) and HPLC peptide maps of the proteolytic products of the deglycosylated form after single digestion with endoproteinase Glu-C (B) and consecutive digestion with endoproteinase Glu-C and proteinase K (C) under nonreducing conditions. (A) The receptor was deglycosylated as described under Experimental Procedures. Analysis was done on a 12% SDS–polyacrylamide gel under nonreducing conditions: (1) glycosylated receptor; the many bands denote a heterogeneous glycosylation; (2) after treatment with *N*-glycosidase F. The weak bands migrating between 14 and 20 kDa are proteolytic products generated during incubation with *N*-glycosidase F. The peptides of the endoproteinase Glu-C and endoproteinase Glu-C/proteinase K digestions were separated by reversed-phase HPLC (B) on an Aquapore RP-300, 220 × 2.1 mm column and (C) on an Aquapore OD-300, 220 × 2.1 mm column (Brownlee-Labs), respectively. Both columns were developed with a linear gradient of 0–65% acetonitrile containing 0.1% trifluoroacetic acid at 0.2 mL/min at room temperature. The absorbance was recorded at 214 nm (mAU, milliabsorbance units). The protein peaks containing the peptides analyzed are indicated with small letters (a–e).

further studied which of the disulfide bonds are essential for activity, by replacing the cysteines with serines using site-directed mutagenesis (Sayers et al., 1988). A group of 13 mutant expression plasmids was constructed using synthetic oligonucleotides. In single mutations, one cysteine was individually substituted with a serine residue. In four double mutations, two successive cysteines were exchanged each time, according to the order of their occurrence from the N-terminus (60, 68; 105, 150; 178, 183; 197, 218). In a quadruple mutation, the four successive cysteine residues 178, 183, 197, and 218, most proximal to the carboxyl-terminus, were replaced.

Wild-type soluble IFNγ receptor and its mutants were expressed in *E. coli*. We used this expression system in order to make use of the advantage of fast fermentation and efficient purification (Stüber et al., 1990). The proteins were isolated from 1-L cell cultures, and as each of them included an auxiliary sequence of six histidine residues at the carboxyl terminus, they were purified by metal-chelate affinity chromatography (Hochuli et al., 1988; Stüber et al., 1990). The receptors were eluted from the column with 8 M urea with a purity higher than 80%.

All mutant receptors comigrated with the wild-type protein under reducing conditions (Figure 3A). Under nonreducing conditions, the wild type and the receptors including two or four mutated cysteine residues, and carrying no free thiol groups, migrated as strong, sharp bands (Figure 3B, lanes 1, 4, 7, 10, and 13–15). The constructs containing one mutated cysteine residue migrated as weaker, mostly diffuse bands, probably due to the free sulfhydryl group (Figure 3B, lanes 2, 3, 5, 6, 8, 9, 11, and 12). The proteins mutated at Cys^{60}, Cys^{68}, Cys^{197}, and Cys^{218} migrated as two equally strong bands (Figure 3B, lanes 2, 3 and 11, 12, respectively). After reduction, these mutants migrated as one band (Figure 3A, lanes 2, 3 and 11, 12). The double signal seen under nonreducing conditions probably represents conformations with scrambled disulfide bonds between the nonmutated cysteine residues. These nonnative conformations were generated during the disruption of the *E. coli* cells. Since they also contain the auxiliary 6×His sequence, they were copurified with the proteins having native conformation. We did not try to separate further the native from the nonnative conformational forms. The 22–25-kDa bands seen in Figure 3B are shorter fragments of the receptor. They were generated due to internal starts of translation within the mRNA. Since they also contain the 6×His sequence, they were copurified with the full-length soluble receptors (Fountoulakis et al., 1991a).

Ligand Binding Capacities of the Mutated IFNγ Receptors. The ligand binding capacities of the mutated proteins were analyzed by ligand blots and were compared to that of the nonmutated receptor (Figure 4A). The receptors mutated at cysteine residues 60, 68, 105, or 150 did not show any ligand binding capacity (Figure 4A, lanes 2, 3 and 5, 6). Those mutated at Cys^{178} or Cys^{183} showed a very weak ligand binding capacity (less than 1% of the wild type; compare lanes 8, 9



Peptide	M_r Calculated	Observed
a	4346.9	4347.4
b	5441.2	5441.2
c	2620.1	*)
d	1436.5	1437.0
e	903.0	903.5

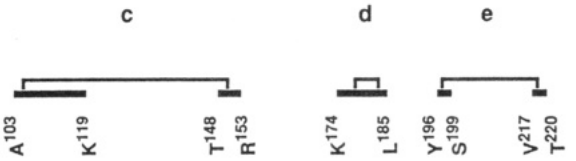


FIGURE 2: Schematic representation of the extracellular domain of the IFNγ receptor. The indicated sequence corresponds to that of the soluble receptor produced in insect cells. The positions of the cysteine residues are denoted by vertical bars and the connectivities by solid horizontal lines. The insect cell-derived protein, after removal of the N-linked oligosaccharides, was digested with endoproteinase Glu-C and endoproteinase Glu-C/proteinase K as described under Experimental Procedures. The letters a–e indicate the analyzed peptides isolated from the corresponding HPLC peaks (Figure 1B,C). (a, b) peptides derived from single digestion with proteinase Glu-C and (c–e) from consecutive digestion with endoproteinase Glu-C and proteinase K. The first and last residues of each sequence are indicated. (Inset) Calculated and observed relative molecular weights (M_r) of peptides a–e. Asterisk, no molecular weight was obtained. Found peptide sequences (the expected positions of the cysteine residues are shown in boldface type; X, unidentified residue; small letters denote residues not identified with certainty): (a) XIDA**X**INISHHY**X**NISDhVGDPSNSLWVRVKARVGQKE. (b) IQYKILTQKEDD**X**DEIQ**X**QLAIPVSSLNSQY**X**VSAE...V**X**-ITIFNSSIKGS. (c) AV**X**RDGKIGPPkLDIRk...TT**X**Ir. (d) KEDD**X**DEIQ**X**QL. (e) Y**X**VS...V**X**IT.

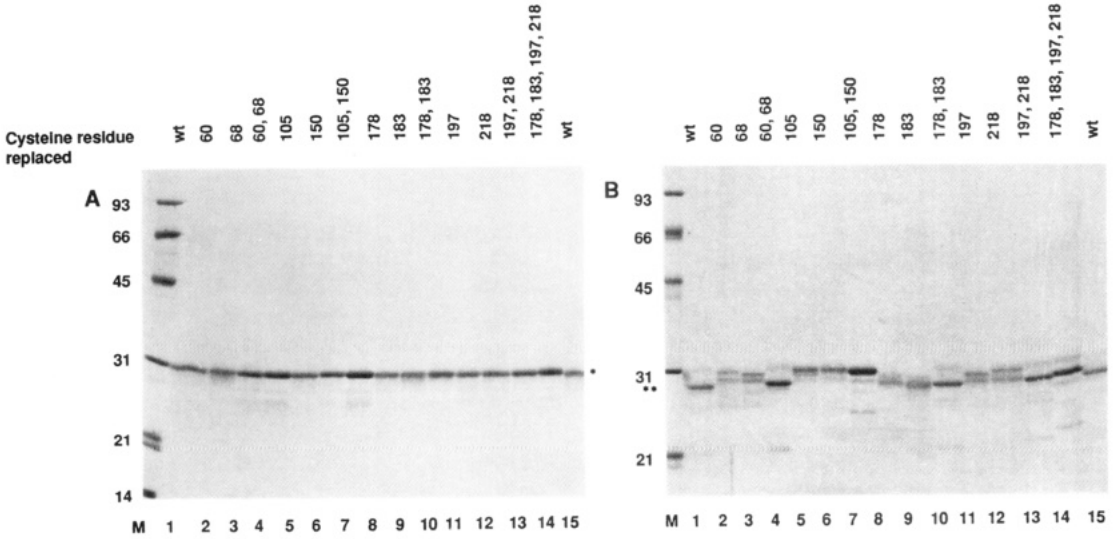


FIGURE 3: SDS–PAGE analysis of the mutated recombinant IFNγ receptors under reducing (A) and nonreducing (B) conditions. The receptors, wild type (wt) and mutated at the indicated cysteine residues, were expressed in *E. coli* and purified by metal-chelate affinity chromatography as described (Stüber et al., 1990); 1.5 μg of protein was loaded in each case. Analysis was done on 12% SDS gels, stained with Coomassie blue R-250. (A) The probes were reduced by addition of 2% β-mercaptoethanol and by boiling for 5 min. (M) protein size markers in kDa. (*) Reduced soluble receptor; (**) nonreduced receptor.

with lanes 1 and 15; Table I). The proteins with replaced Cys^{197} or Cys^{218} had a higher residual activity, about 3% of that of the nonmutated receptor (Figure 4A, lanes 11 and 12). The double mutant at cysteines-60 and -68 showed no activity (lane 4). The proteins carrying double mutation at cysteines-105 and -150, -178 and -183, or -197 and -218 (Figure 4A, lanes 7, 10 and 13, respectively) showed a reduced ligand binding capacity, 5–15% in comparison with the nonmutated receptor. The quadruple mutant, in which the four cysteine residues at the carboxyl-terminus region were replaced, did not show ligand binding capacity (Figure 4A, lane 14). Thus, the ligand blot analysis showed that all cysteine residues are required for binding.

Comparison of the ligand binding capacities of the wild-type and mutant receptors assumes that all proteins fold in a similar way on the nitrocellulose strips during the perfor-

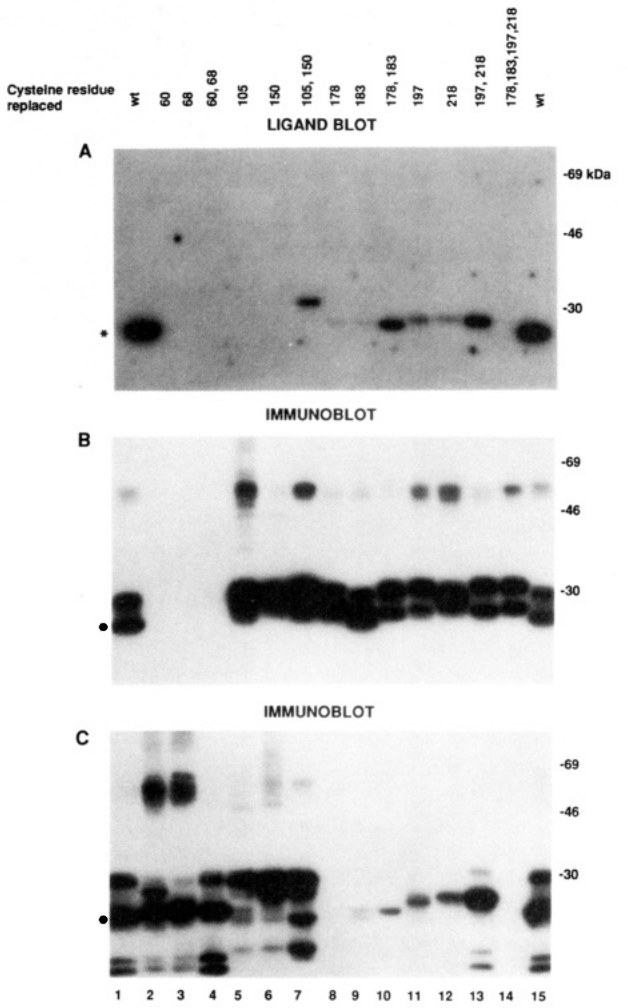


FIGURE 4: Ligand blot (A) and immunoblot (B, C) analyses of the mutated recombinant IFNγ receptors. The mutated receptors were engineered, expressed in *E. coli*, and purified as stated under Experimental Procedures; 1.5 μg of protein was loaded in each case. The proteins, wild type (wt) and mutated at the indicated cysteine residues, were electrophoresed under nonreducing conditions on 12%–SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes. The membranes were probed with ^{125}I-IFNγ (A), antibody γR38 (B), or antibody γR99 (C), followed, in the two last cases, by incubation with ^{125}I-labeled sheep anti-mouse Ig. (*) 27-kDa IFNγ binding band of the nonmutated receptor.

mance of the ligand blots. In folding studies, the wild-type and the double mutant receptors showed a similar behavior (unpublished results). Thus, the reduced ligand binding capacities of the double mutants (Figure 4A, lanes 7, 10, and 13), in comparison with the wild type (Figure 4A, lanes 1 and 15), are due to the lack of essential disulfide bonds and not to the inability of the mutants to refold properly.

Capacity of Antibodies To Detect the Mutated IFNγ Receptors. We further investigated whether the mutated soluble IFNγ receptors are recognized by monoclonal antibodies raised against the native protein. The antibody γR38 recognized all mutated receptors (Figure 4B, lanes 1 and 5–15), except those carrying replacements at Cys^{60} and Cys^{68} and at both positions simultaneously (Figure 4B, lanes 2–4). Comparison of the SDS–PAGE, ligand blot, and immunoblot analyses revealed that this antibody detected a non-IFNγ binding 30-kDa form with higher affinity than the binding form of the receptor (Figures 3B and 4A,B, lanes 1 and 15). As we previously described (Fountoulakis et al., 1990, 1991a), the 30-kDa protein represents a receptor form with wrong shuffling of disulfide bonds, present only in the preparations

Table I: Interaction between IFNγ and Anti-Receptor Antibodies with Mutated IFNγ Receptors[a]

changed cysteine residue	% binding of mutated IFNγ receptors to		
	IFNγ	antibody γR38	antibody γR99
none	100	100	100
60	0	0	100
68	0	0	100
60, 68	0	0	100
105	0	100	70
150	0	100	100
105, 150	5	100	100
178	~1	100	0
183	~1	100	2
178, 183	10	100	5
197	~3	100	15
218	~3	100	20
197, 218	15	100	100
178, 183, 197, 218	0	100	0

[a] The ligand binding capacities of the mutated receptors were estimated by ligand blots and were expressed as a percentage of that of the nonmutated protein (=100%). The capacities of the antibodies to bind the mutated receptors were determined by immunoblots (binding of the wild type =100%).

of the *E. coli*-derived receptor and absent in the preparations of the insect cell-derived soluble receptor. Antibody γR38 also detected 30–32-kDa non-IFNγ binding forms of the proteins mutated at Cys^{178}, Cys^{183}, Cys^{197}, and Cys^{218}, of the double mutants at cysteines-178, -183, and -197, -218, and of the quadruple mutant, with higher affinity than the IFNγ binding forms of the receptor (Figure 4A,B, lanes 8–14). These signals also represent mutated proteins with wrong or disrupted disulfide bonds. The nonnative receptor forms were recovered in solution after removal of the denaturants used during purification (Fountoulakis et al., 1990).

The antibody γR99 detected mutants with substituted cysteine residues at positions 60, 68, 105, or 150 and the proteins with double mutation at Cys^{60} and Cys^{68}, or Cys^{105} and Cys^{150} (Figure 4C, lanes 1–7). It did not recognize the receptor with substituted Cys^{178} (lane 8). It detected weakly the constructs mutated at Cys^{183}, Cys^{197}, or Cys^{218} (Figure 4C, lanes 9, 11, and 12, respectively) and also the double mutant at cysteines-178 and -183 (lane 10). This antibody bound very efficiently the construct in which both Cys^{197} and Cys^{218} residues were replaced (lane 13). It did not bind the mutant where the four cysteine residues 178, 183, 197, and 218 were substituted (lane 14). The antibody γR99 recognized several bands migrating between 22 and 25 kDa (Figure 4C, lanes 1–7, 13, and 15), representing products of internal translation starts, that lack the IFNγ binding region near the amino terminus (Fountoulakis et al., 1991a). These polypeptides were not detected by either radiolabeled IFNγ (Figure 4A) or antibody γR38 (Figure 4B). Both antibodies γR38 and γR99 detected dimeric forms (55-kDa bands) of some of the mutants (Figure 4B,C).

The immunoblots showed that the specific antibodies γR38 and γR99 recognize conformational epitopes stabilized by disulfide bonds between cysteine residues 60 and 68, and 178 and 183, respectively. The site-directed mutagenesis of the cysteine residues helped to further restrict the length of the conformational epitope recognized by antibody γR38 (Garotta et al., 1990). The epitope detected by this antibody is not stabilized by the disulfide bond in which Cys^{105} participates and probably does not include this residue, being restricted in the region 9–105. Cysteines-105 and -150 do not contribute to stabilization of the epitope detected by antibody γR99. This antibody, however, requires sequences to the left of these residues for binding (Garotta et al., 1990), the epitope being

most likely confined to a region between residues 53 and 193. The polyclonal antibody R3891 efficiently recognized the wild type and the proteins with double mutations at cysteines-60 and -68, -105 and -150, and -197 and -218, respectively, and the quadruple mutant. The proteins with single cysteine mutations and the double mutant at residues 178 and 183 were weakly detected (data not shown).

DISCUSSION

Previous studies with the native IFNγ receptor have shown that at least one disulfide bond is critical for its binding capacity and the compactness of the protein (Fountoulakis et al., 1989, 1990). Here, we demonstrate that the cysteine residues of the extracellular domain of the native sequence form four consecutive disulfide bonds, all of them essential for full ligand binding capacity. The analysis of unfolding intermediates of the IFNγ receptor had also revealed that all four disulfide bonds should be intact in order to have ligand binding (Fountoulakis, 1992). The alignment of the disulfide bonds reported here is essential for the study of the folding pathway of the IFNγ receptor. Investigation of the folding mechanism of this membrane-anchored protein may contribute to a better understanding of the general protein folding problem.

The ligand binding domain of the IFNγ receptor and the structure of IFNγ have been previously studied. Taking those findings into consideration, as well as the results of this study, we can summarize our present knowledge about the ligand–receptor interaction: (i) the antibody γR38 detects a conformational epitope stabilized by the disulfide bond Cys^{60}–Cys^{68}, and this antibody does not precipitate a ligand–receptor complex (Garotta et al., 1990); (ii) the antibody γR99 detects a conformational epitope stabilized by the disulfide bond Cys^{178}–Cys^{183}, and it precipitates a ligand–receptor complex (Garotta et al., 1990); (iii) almost the whole extracellular domain of the receptor (including the polypeptide stretches close to both termini) and the disulfide bonds are required for full ligand binding capacity (Ozmen et al., 1990; Fountoulakis et al., 1991a); (iv) the stretch 1–150 of the IFNγ receptor includes four essential cysteine residues and is an exposed region because many proteolytic cleavage sites on this domain were utilized, in particular, the cleavage site Glu^{93} by endoproteinase Glu-C, and it carries three potential glycosylation sites; (v) the two subunits of IFNγ dimer have six α-helices each and possess an antiparallel orientation. Most likely, the N-terminus of the one subunit and the C-terminus of the other subunit are involved in binding (Ealick et al., 1991);(vi) two molecules of the soluble receptors produced in insect and Chinese hamster ovary cells interact with one IFNγ dimer (Fountoulakis et al., 1992b, 1993); (vii) a high hydrodynamic radius was measured for the ligand–receptor complex, suggesting an open structure and probably deviation from globular shape.

The IFNγ receptor belongs to the group of single-chain, monomeric receptors with one transmembrane region (Aguet et al., 1988). The intracellular domain is phosphorylated at serine/threonine residues (Khurana Hershey et al., 1990; Mao et al., 1990). Tyrosine phosphorylation has not been reported so far. In spite of low homology between the IFNγ receptor and the other known cytokine receptors, structural analysis of its sequence suggested that the IFNγ receptor belongs to the superfamily of receptors of hematopoietic factors and growth hormones. The members of this superfamily are subdivided in two classes. Class I includes the receptors for interleukins-2, -3, -4, -6, and -7, granulocyte and granulocyte macrophage–colony stimulating factors, erythropoietin, prolactin and growth hormone (GRH). Class II comprises the receptors for IFNα/β, IFNγ, and tissue factor, the cell receptor for the coagulation protease factor VII (Bazan, 1990a). According to this analysis, the extracellular segments of class I and II receptors show repeated homologous modules of 200 and 210 amino acids, respectively, and each module includes a top and a bottom domain, each about 100 residues in length. Predictive structure analysis locates seven β-strands in conserved regions of each domain. The β-strands form two sheets that fold to antiparallel β-sandwiches with a topology similar to immunoglobulin constant domains. Top and bottom domains are linked by a hydrophobic hinge region in a V-shaped structure. This trough at the converge of the two domains most likely forms the ligand binding site of the receptors (Bazan, 1990a). The top domain of class I receptors is characterized by a conservation of four sequentially linked cysteines, while both the top and bottom Ig-like domains of class II receptors show one distinctive cysteine pair. In both class I and class II receptors, the other cysteine residues seem to be scattered.

The members of the hematopoietic cytokine receptor superfamily possess a conserved array of sequentially linked cysteines that is notably dissimilar to the pattern found in the IFNγ receptor. The cell-surface molecule tissue factor is a closer homologue of the IFNγ receptor (Bazan, 1990a). The extracellular domain of this 219-residue-long molecule includes 4 cysteine residues at positions 49, 57, 186, and 209 (Nemerson, 1988), probably sequentially linked, i.e., Cys^{49}–Cys^{57} and Cys^{186}–Cys^{209}. These disulfide bonds are almost identical with the bonds Cys^{60}–Cys^{68} and Cys^{197}–Cys^{218} of the IFNγ receptor. These disulfide bonds involve cysteines that are also conserved in the extracellular segment of the IFNα/β receptor (2 modules with a total of 409 residues) and the mouse IFNγ receptor and are required for IFNγ receptor function (Bazan, 1990b). The Cys^{197}–Cys^{218} disulfide bond is also essential for full activity, although a residual activity is present after mutation of the corresponding cysteine residues (Figure 4A). The successive disulfide links are different in sequence between hematopoietic receptors, IFNγ receptor/tissue factor, and receptors that are "classical" members of the Ig superfamily, like the receptors for platelet-derived growth factor, interleukin-1, and others (Williams, 1989; Sims et al., 1988) that have binding segments constructed of Ig repeats.

The two-domain structure and the presence in each domain of two β-sheets of such a class I receptor were confirmed by crystallographic analysis of the GRH–GRH receptor complex (de Vos et al., 1992). The topology of the sandwich was not that of the immunoglobulin constant domain, as here a "sheet switching" has taken place. On the basis of these data and the results presented in this paper, one can predict the common architecture of class I receptors (i.e., GRH receptor) and class II receptors (i.e., IFNγ receptor). The disulfide links determined for the IFNγ receptor are compatible with the sequence alignment of Bazan and the suspected similarity to the GRH receptor. The Cys^{60}–Cys^{68} disulfide bond of the IFNγ receptor is located at the top Ig-like module and probably links two adjacent β-strands. This link corresponds to the disulfide bond 83–94 of the GRH receptor. The two last disulfide bonds, Cys^{178}–Cys^{183} and Cys^{197}–Cys^{218}, are located at the bottom Ig-like module of the IFNγ receptor and have no counterparts in the GRH receptor. The Cys^{178}–Cys^{183} link probably connects the ends of strands D′ and E′ [nomenclature of the strands according to Bazan (1990a)] between which a disulfide bond is structurally possible. The

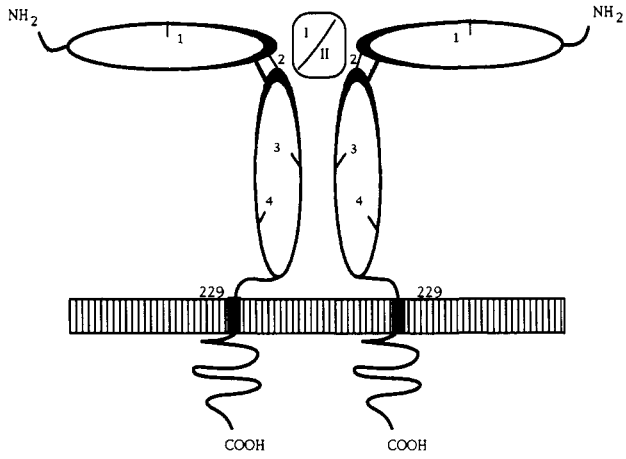


FIGURE 5: Schematic representation of the human IFNγ–IFNγ receptor complex and mode of their binding. The model was based on the data presented under Discussion and was drawn in analogy to the crystal structure of the growth hormone–growth hormone receptor complex (de Vos et al., 1992). The two Ig-like domains of the receptor and the approximate location of the disulfide bonds are indicated. The approximate ligand binding site at the junction of the two domains is denoted by a broad line. The two subunits of IFNγ are indicated I and II. 1, 2, 3, and 4 denote the disulfide bonds Cys^{60}–Cys^{68}, Cys^{105}–Cys^{150}, Cys^{178}–Cys^{183}, and Cys^{197}–Cys^{218}, respectively. 229 shows the residue number of the extracellular domain of the IFNγ receptor.

disulfide bond 197–218 would connect the two cysteines located in the middle of the adjacent strands F′ and G′ which seem to be spatially close. Cysteine residue 105 is located at the top domain and cysteine-150 at the bottom domain of the IFNγ receptor, both in the area of the interdomain interface. Thus, the disulfide bond Cys^{105}–Cys^{150} would connect the two Ig-like domains and is essentially responsible for the compactness of the molecule. Our results confirm this hypothesis. The receptors mutated at Cys^{105} and Cys^{150} and the protein with double mutations at the same residues showed a significant shift in mobility on nonreducing SDS–polyacrylamide gels. In comparison with the nonreduced wild type (27 kDa), these mutants migrated at about 32 kDa like the reduced wild-type receptor. The GRH receptor has not such an interdomain disulfide link. Figure 5 shows the approximate location of the four disulfide bonds on the two domains of the IFNγ receptor.

Assuming that the ligand binding domain is conserved in class I and class II cytokine receptors and in analogy to the GRH–GRH receptor complex (de Vos et al., 1992), we suggest that the sequences of the loops connecting β-strands A and B, and E and F of the top Ig-like module and the sequences connecting strands B′ and C′, and F′ and G′ of the bottom Ig-like module of the receptor are involved in ligand binding. Thus, the ligand binding site of the IFNγ receptor is probably the exposed interface region between top and bottom Ig-like domains (Figure 5). The ligand binding pocket probably requires amino acid stretches from different parts of the receptor for stabilization, which explains the requirement of almost the whole extracellular domain for full activity. Consequently, the disulfide bonds are essential for ligand binding, as they stabilize the conformation of the domains. The proposed model explains why antibody γR38, which detects a conformational epitope on the top domain of the IFNγ receptor, on account of steric hindrance, does not precipitate the receptor bound to IFNγ, whereas antibody γR99, which detects an epitope located on the bottom Ig-like domain, is not hindered and precipitates the ligand–receptor complex.

Two receptor molecules bind one IFNγ dimer (Figure 5). The evidence for this binding mode is based on data found with the insect cell- and Chinese hamster ovary cell-derived soluble receptors (Fountoulakis et al., 1992b, 1993) and has not yet been confirmed for the cellular receptor. Other, not yet identified protein(s) seem(s) to bind the ligand–receptor complex and to be involved in signal transduction (Gibbs et al., 1991; Hibino et al., 1992; Hemmi et al., 1992). Crystallization studies are in progress to elucidate in detail the structure of the ligand–receptor complex and to precisely define the interaction site.

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