Structural analysis of the human interferon γ receptor: A small segment of the intracellular domain is specifically required for class I major histocompatibility complex antigen induction and antiviral activity

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ABSTRACT Mutations of the human interferon γ (IFN- γ) receptor intracellular domain have permitted us to define a restricted region of that domain as necessary for both induction of class I major histocompatibility complex antigen by IFN- γ and protection against encephalomyocarditis virus. This region consists of five amino acids (YDKPH), all of which are conserved in the human and murine receptors. Tyr-457 and His-461 are essential for activity. Approximately 80% of the amino acids of the intracellular domain of the receptor is not required for major histocompatibility complex class I antigen induction or for antiviral protection against encephalomyocarditis virus. The observation that there was no protection by IFN- γ against vesiculostomatitis virus indicates that other factors, in addition to chromosome 21 accessory factor(s), are required to generate the full complement of transduction signals from the human IFN- γ receptor.

Interferons (IFNs) induce a number of characteristic antiviral, antiproliferative, and immunomodulatory effects by interacting with specific cell-surface receptors (1, 2). IFNs α , β , and ω bind to one type (IFN- $\alpha/\beta/\omega$) of receptor, whereas IFN- γ binds to a distinct receptor (IFN- γ R) (1–3). Both types of receptors bind IFNs with high affinity (K_d of 1×10^{-9} to 1×10^{-11} M) and are present in relatively low numbers (usually <20,000 per cell) in a wide variety of cell types.

After binding its ligand, the IFN- γ R is internalized and eventually recycles back to the membrane (4). For the IFN- γ R, these events are accompanied or followed by a number of processes thought to be involved in signal-transduction mechanisms (1, 2, 5, 6), such as activation of the Na^+/K^+ antiporter (7), stimulation of Ca^{2+} and phospholipid-dependent protein kinase C (8), and alterations in the adenylate cyclase and G protein system (9, 10).

Our results with somatic cell hybrids demonstrated that the IFN- γ R is not sufficient for a biologically functional ligand-receptor complex (11): in human cells, a factor encoded by chromosome 21 is required (12), and in murine cells, a factor encoded by chromosome 16 is necessary (13). To date, these human and murine “accessory factors” have not been identified or characterized. However, experiments with human/murine chimeric receptors indicate that the accessory factors interact with the extracellular domain (14–16).

Although the extracellular domain is clearly involved in ligand binding and interaction with the accessory factor, there is little understanding of the role of the intracellular domain in IFN signal transduction. In the present study, we examined the structural requirements of the human IFN- γ R (Hu-IFN- γ R) intracellular domain for biological activity. Specific mutations of the intracellular domain have enabled

us to map the structural elements required for class I major histocompatibility complex (MHC) antigen induction and antiviral activity. The results of this mutational analysis are presented here; preliminary results have appeared in abstract form (17, 18).

MATERIALS AND METHODS

Cells and Plasmids. The hamster/human somatic cell hybrid 153B7-8 was derived and maintained as described (14, 19) in F-12D medium (GIBCO), which is deficient in hypoxanthine, containing 5% fetal bovine serum. The 153B7-8 cells had been transfected with a genomic clone for the class I HLA-B7 antigen (19, 20) and contain human chromosome 21q. The expression vectors pLEN (21) and pRSVneo (22) were described elsewhere. Construction of the expression vector pVJ2-H γ R from the Hu-IFN- γ R cDNA (23, 24) has been reported (19, 25). The plasmid pGEM-4 was obtained from Promega.

Reagents, Restriction Endonucleases, and Other Enzymes. Human IFN- γ (Hu-IFN- γ), with a specific activity of 1.1×10^7 units/mg, was isolated from *Escherichia coli* (26). Human IFN- $\alpha/\text{D}(\text{Bgl})$ was prepared as reported (27). Restriction endonucleases were obtained from New England Biolabs and Boehringer Mannheim. The Klenow fragment of DNA polymerase I and G418 were purchased from GIBCO/BRL. *Taq* polymerase was a product of Perkin-Elmer. Bal-31 was from Boehringer Mannheim.

Construction of Mutants. The plasmid pVJ2-H γ R444 was constructed by PCR-directed mutagenesis. A primer containing an *Xho* I site and a stop codon was used to amplify the cDNA sequences corresponding to amino acids 1–444. It should be noted that amino acid positions of the Hu-IFN- γ R are designated with respect to the initial methionine of the precursor protein as position 1 (23, 24). The PCR-generated DNA was ligated and then cut with *Xho* I (28) and cloned into eukaryotic expression vector pVJ2 (19) at the *Xho* I site (25). This plasmid deletes 45 amino acids from the carboxyl terminus of the Hu-IFN- γ R.

The plasmids pHT-11 and pHT-25 were prepared by oligonucleotide-directed mutagenesis. Termination codons were introduced, resulting in premature stop codons at positions 483 and 480, respectively. The oligodeoxynucleotides were treated with kinase, hybridized, and ligated to a 5'-flanking *Mbo* II–*Bst*EII fragment and to a 3'-flanking *Bst*XI–*Fok* I fragment. This product was then subcloned into pGEM4 containing the Hu-IFN- γ R cDNA that had been digested with restriction endonucleases *Bst*XI and *Bst*EII.

Abbreviations: IFN, interferon; Hu-IFN- γ , human IFN- γ ; IFN- γ R, IFN- γ receptor; Hu-IFN- γ R, human IFN- γ R; MHC, major histocompatibility complex; EMCV, encephalomyocarditis virus; VSV, vesicular stomatitis virus.

Plasmids pHT-18 and pHT-29 resulted from spontaneous deletions occurring during the construction of pHT-11 and pHT-25. All other constructs introducing premature termination codons were assembled as in Fig. 1.

Plasmid pD-1 was constructed by restriction endonuclease digestion of the cDNA with *Bst*XI. The ends were digested for short periods of time (up to 10 min) with *Bal*-31. The plasmid containing the largest of these deletions was designated pD-1; in pD-1 residues 307–418 are deleted. The pD-2 plasmid was constructed by PCR with oligonucleotide primers that deleted the region encoding amino acids 419–456. The PCR-amplified DNA was digested with *Nsi* I and cloned into the cDNA that had been cut with *Nsi* I. Plasmid pD-3 substitutes Trp⁴⁵⁷ for Tyr⁴⁵⁷; it was constructed as shown in Fig. 1, except that both primers encoded the amino acid substitution rather than a termination codon. Sequences of all constructs were confirmed by the dideoxynucleotide chain-termination sequencing procedure (29).

Expression Vectors, Transfection, and Cytofluorography. All mutant cDNAs (except that used to construct pVJ2-H γ R444) were excised from pGEM4 with *Bam*HI and *Hind*III. The ends were filled in with the Klenow fragment of DNA polymerase I, and the DNA was subcloned into expression vector pLEN (21). Expression was driven by simian virus 40 (pVJ2-

FIG. 1. Procedure for introducing premature termination codons into the Hu-IFN- γ R cDNA. Briefly, Hu-IFN- γ R cDNA was amplified with two sets of primers with one primer present at a 100-fold lower concentration. One primer (primer 3) encoded a premature termination codon. (Occasionally, both primers 2 and 3 contained a premature termination codon.) The single-stranded products from steps 1 and 2 were added to a third PCR reaction, and a double-stranded DNA product was amplified by primers 1 and 4. This product was digested with *Bst*XI and *Bst*EII and cloned back into the cDNA clone that had been digested with the same enzymes. For expression, the cDNA insert was excised with *Bam*HI and *Hind*III and subcloned into pLEN. Expression vector pLEN has been described (21).

H γ R444) or metallothionein (pLEN-based vectors) promoters. The expression vectors were transfected into recipient 153B7-8 cells by the calcium phosphate method (30). Twenty micrograms of pVJ2-H γ R444 vector was used per 500,000 cells. Twenty micrograms of the pLEN constructs plus 2 μ g of pRSVneo were cotransfected by the same procedure. Colonies were selected in antibiotic G418 at 400 μ g/ml. Cells were assayed for class I MHC antigen expression after a 48- to 72-hr incubation with Hu-IFN- γ at 2500 units/ml. MHC class I antigen expression was quantified with mouse monoclonal antibody W6/32 (31) and fluorescein isothiocyanate-labeled goat anti-mouse IgG (Sigma). Cells were analyzed on a Coulter Epics Profile cytofluorograph with a 15-mW argon laser tuned to 488 nm, as reported (19, 20). For each sample, 10,000 events were accumulated, and data were analyzed with Cytologic software. All constructs were assayed in pooled transfectant cultures; however, when pooled transfectants were negative for MHC class I antigen induction, several clones were isolated, grown, and assayed individually. In all experiments, a cell line transfected with the complete cDNA (pHT-8 or pVJ2-H γ R) was used as a positive control (19, 20, 25). Cells scored positive for MHC induction had a ratio (logarithm of relative fluorescence with IFN- γ /logarithm of fluorescence without IFN- γ) of at least 1.2. Cells scored negative had a ratio of 1.00 ± 0.05 .

Binding Assay. Binding of Hu-IFN- γ to transfected cells was done as described with ³²P-labeled IFN- γ (13). Criterion for positive binding was at least a 2-fold ratio of counts without unlabeled competitor to counts with 100-fold excess of unlabeled competitor.

Antiviral Assay. Transfected cells were assayed for resistance to vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) by a cytopathic effect inhibition assay (32). In general, the magnitude of the antiviral effect for positive cells was similar to that obtained with cells transfected with complete Hu-IFN- γ R. An exception was noted with cells that were transfected with pD-2; the antiviral activity of these cells was very low. Negative cells exhibited no antiviral protection at any IFN- γ concentration.

RESULTS

To assay the truncation and deletion mutants of the intracellular domain, we used a Chinese hamster ovary (CHO) somatic cell hybrid line containing a translocated human chromosome 21q, the chromosome arm that contains the gene(s) encoding the accessory factor(s) required for IFN- γ function; this cell line is designated 153B7-8 (19, 20, 25). These cells were also stably transfected with the human MHC class I HLA-B7 gene (19, 33), so that all that is required for MHC class I induction is a functional Hu-IFN- γ R and the ligand. Fig. 2 shows that 153B7-8 cells do not respond to IFN- γ , nor do cells transfected only with pRSVneo. Cotransfection of these cells with plasmids pRSVneo and pHT-8 (plasmid pLEN containing the complete Hu-IFN- γ R cDNA) enables MHC induction in response to Hu-IFN- γ .

To determine the structural requirements of the intracellular domain of the Hu-IFN- γ R for signal transduction, we constructed a number of mutants containing premature termination codons. These mutants were engineered either by PCR or by cloning oligonucleotides containing termination codons. Two constructs were obtained by spontaneous mutations that occurred during the cloning process. When cells transfected with plasmid pVJ2-H γ R444 were assayed, no MHC class I antigen induction was seen in pooled transfectant cultures or in subclones of these cultures (Figs. 2D, and 3). Because this construct produced receptors that are truncated by 45 amino acids at the carboxyl-terminal end, at least some portion of the terminal 45-amino acids of the protein

FIG. 2. Cytofluorography of transfectants. Pooled transfectants were incubated with mouse monoclonal antibody W6/32 (31) against human HLA class I antigens and subsequently with fluorescein isothiocyanate-labeled goat anti-mouse IgG. (A) Cell line 153B7-8, which contains a transfected MHC gene (19, 20) and human chromosome 21q. (B) Cell line 153B7-8 transfected with pRSVneo (containing a neomycin resistance gene driven by a simian virus 40 promoter). (C) Cell line 153B7-8 cotransfected with pHT-8 (Hu-IFN- γ R cDNA) and pRSVneo. (D) Cell line 153B7-8 cotransfected with pVJ2-H γ R444, which deletes 45 carboxyl-terminal amino acids. Solid line, control; stippled area, Hu-IFN- γ at 2000 units/ml.

must perform a function critical to signal transduction (17, 18).

To define this region further, we constructed terminator mutants that progressively truncated the protein from the carboxyl-terminal end. Positive responses to Hu-IFN- γ were scored by cytofluorographic analysis, as illustrated in Fig. 2C. The results obtained with these constructs (Fig. 3) indicated that 28 of the terminal 45 amino acids are not

Construct	COOH-Terminal Amino Acid	IFN Bound	MHC Ind.	Anti-EMC
pHT-8	Ser ⁴⁸⁹	+	+	+
pHT-11	Thr ⁴⁸²		+	
pHT-25	Thr ⁴⁷⁹		+	
pHT-29	Ser ⁴⁷⁵		+	
pHT-18	Lys ⁴⁷³		+	
pHT-34	Leu ⁴⁶⁶	+	+	+
pHT-1	Asp ⁴⁶⁵		+	
pHT-2	Val ⁴⁶⁴		+	
pHT-5	His ⁴⁶¹	+	+	+
pHT-6	Pro ⁴⁶⁰	+	-	-
pHT-19	Gly ⁴⁵⁶	+	-	-
pHT-28	Ile ⁴⁴⁹	+	-	-
pVJ2-H γ R 444	Glu ⁴⁴⁴	+	-	-

FIG. 3. Biological activity of constructs with premature termination codons. Constructs with premature termination codons were transfected, and resistant colonies were selected with G418. Pooled transfectants were assayed for Hu-IFN- γ binding, HLA-B7 antigen induction (ind.), and antiviral activity against EMCV (EMC). In those instances where HLA-B7 antigen induction and antiviral activity were not seen, subclones of the pooled transfectants were also assayed with the same result (data not shown). +, Activity; -, no activity; no designation, not done. The intact Hu-IFN- γ R expression vector is designated pHT-8. ECD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain.

required for MHC class I antigen induction or antiviral activity. The last functional truncation, mutant pHT-5, deletes 28 amino acids from the carboxyl terminus of the protein, whereas pHT-19 deletes 33 amino acids and results in a nonfunctional receptor. This result indicated that the segment of amino acids required for signal transduction could be located between amino acids 461 and 457 (inclusive). Fig. 3 shows that the plasmid pHT-6, which replaces the His⁴⁶¹ codon with a termination codon, produced a truncated receptor incapable of inducing MHC class I antigen expression or antiviral activity. This result, therefore, indicates that His⁴⁶¹ is one of the residues required for Hu-IFN- γ R activity.

In addition to its ability to induce class I MHC antigen expression, Hu-IFN- γ induces cytoprotective effects against viral infection. Table 1 shows that the CHO cell system used to assay MHC class I antigen induction was found to be functional in terms of protection against EMCV. The presence of both the Hu-IFN- γ R cDNA and human chromosome 21q was required for this effect. The same cells could not inhibit VSV infection in the presence of Hu-IFN- γ . As a control, Hu-IFN- α /D (Bgl), an IFN- α active on hamster cells (19, 27), was shown to stimulate protection against EMCV and VSV. Assay of the premature termination mutants in this system showed that antiviral effects against EMCV were lost at the same point that MHC class I antigen inducibility was lost (Fig. 3). This result also demonstrates that the chromosome 21 factor, which we discovered (11, 12) and which is required for MHC class I induction, also mediates protection against EMCV; however, this factor is not in itself sufficient for generating protection against VSV.

Assay of several subclones from the pooled transfectant cell lines obtained with plasmids pHT-19, pHT-28, and pHT-6 indicated that all subclones that bound IFN- γ were inactive for MHC class I antigen induction and antiviral activity (data not shown). Therefore, the inability of these clones to transduce the Hu-IFN- γ signal is not the result of underrepresentation of receptor-positive clones in the pooled transfectant cultures.

Because mutants with premature termination codons can define only the carboxyl-terminal extent of the region required for MHC class I antigen induction and antiviral activity, internal deletions were made extending from residue 307 to the critical region near the carboxyl terminus (Fig. 4). The first of these deletions, plasmid pD-1, deleted from amino acid 307 to amino acid 418 (inclusive), a 112-amino acid deletion. This mutant was found to be functional in terms of ligand binding, class I MHC antigen induction, and antiviral activity, a result indicating that the 112 amino acids removed apparently have no role with regard to these IFN actions.

To determine more accurately the boundary of the critical region, two other mutants were engineered (Fig. 4). The plasmid pD-2 deletes amino acids 419-456; it produced a receptor that is active in terms of ligand binding, class I MHC

Table 1. Antiviral effects of IFNs with transfected CHO cells

Cell line	cDNA transfected	IFN	Virus	
			EMCV	VSV
CHO-K1	Hu-IFN- γ R	Hu-IFN- γ	-	-
153B7-8	None	Hu-IFN- γ	-	-
153B7-8	Hu-IFN- γ R	Hu-IFN- γ	+	-
153B7-8	Hu-IFN- γ R	Mu-IFN- γ	-	-
153B7-8	Hu-IFN- γ R	Hu-IFN- α /D(Bgl)	+	+

Assays were done as described. +, Significant protection of cells against virus killing in response to the IFN; -, no observable protection. CHO-K1 is the parental Chinese hamster cell line; 153B7-8 cells are CHO-K1 cells containing a transfected MHC antigen gene (HLA-B7) and human chromosome 21q (19, 20). cDNAs transfected into cell lines are shown. IFNs were present at 200-350 units/ml in the first well before serial dilution. Mu, murine.

FIG. 4. Internal deletions and point mutation of the intracellular domain of Hu-IFN- γ R cDNA. The plasmids encoding deletions of amino acids 307–418 and 419–456 (pD-1 and pD-2, respectively) and the plasmid replacing Tyr⁴⁵⁷ by Trp⁴⁵⁷ (pD-3) were transfected, and cells resistant to G418 were assayed for Hu-IFN- γ binding, class I MHC antigen induction (Ind.), and resistance to EMCV (EMC). For pD-3 transfectants, individual clones were also assayed, and all clones that bound Hu-IFN- γ were inactive for MHC antigen induction and antiviral resistance (data not shown). +, Activity; -, no activity; no designation, not done; ECD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain.

antigen induction, and antiviral protection. The plasmid pD-3 has a Tyr-457 $\rightarrow$ Trp substitution. This residue was thought important because tyrosine is known to be a potential target for phosphorylation, and the mutant receptor encoded by pHT-19 (Fig. 3) replaced this tyrosine by a termination codon and was inactive with respect to MHC antigen induction and EMCV resistance. Transfectants expressing this mutant receptor could not bind Hu-IFN- γ , but class I MHC antigen could not be induced, nor was there any detectable antiviral activity in response to Hu-IFN- γ . Several subclones containing pD-3 displayed similar phenotypes.

DISCUSSION

Our results show that a very small region of the Hu-IFN- γ R intracellular domain is required for both class I MHC antigen induction and antiviral activity (Fig. 5). A 37-amino acid section of the intracellular domain, proximal to the transmembrane region, was not examined in these studies but was reported to contain a consensus sequence that is probably required for receptor internalization (34). The mutant receptor produced by construct pD-1 lacks 112 amino acids (residues 307–418) after this consensus sequence and yet is active. Because amino acid 307, a leucine considered a constituent of the consensus sequence for receptor internalization, is replaced by the leucine at position 419 in plasmid pD-1, this mutation would not be expected to disrupt receptor internalization. Because the mutant is active, the amino acids immediately on the carboxyl side of the internalization sequence clearly do not contribute to the activity of the

FIG. 5. Location of critical amino acid sequence in the intracellular domain of Hu-IFN- γ R. Mutations detailed in Figs. 3 and 4 indicate that the five amino acids shown are implicated in biological function of Hu-IFN- γ R. Our results show that Tyr⁴⁵⁷ and His⁴⁶¹ are required for both class I MHC antigen induction and EMCV resistance. The N-terminal methionine of the precursor protein is designated as the first amino acid, and Ser¹⁵, therefore, corresponds to the initial amino acid of the mature receptor. ECD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain.

Hu-IFN- γ R. Another deletion construct, pD-2, produces a deletion from residue 419 to 456 (inclusive); this receptor is also active. In addition, mutants with truncations beginning near the carboxyl terminus and extending toward the transmembrane domain show that 28 amino acids at the carboxyl terminus are not required for the activities that we assayed. Thus, >80% of the intracellular domain cannot be attributed a function based upon our experiments. Various regions within these "dispensable" sections are highly conserved between the human and murine receptors. For example, regions bounded by and including residues 316 and 332, 376 and 405, and 469 and 489 are at least 60% conserved between human and mouse receptors at the amino acid level (23, 24). This suggests that there is some structural or functional significance of these sequences. We shall assay other IFN-induced activities to determine what functions, if any, the rest of the intracellular domain may serve. While this paper was in preparation, Farrar *et al.* (34) reported that two large regions of the Hu-IFN- γ R intracellular domain are required for biological activity. After removing residues 321–396 and 397–450, activity was maintained. Residues 273–320 and 451–489 were required for MHC class I antigen induction. The observation that residues 321–450 are not contributory with regard to class I MHC antigen induction by Hu-IFN- γ is consistent with our data with plasmids pD-1 and pD-2, which showed that amino acids 307–456 are expendable with respect to MHC class I antigen induction and antiviral activity (Figs. 4 and 5).

Earlier studies reported that the Hu-IFN- γ R becomes phosphorylated after ligand exposure (35, 36). The phosphorylation was specific, was proportional to the increase in class I MHC antigen induction, and occurred on serine and threonine residues. On the other hand, our deletions indicate that most potential phosphorylation sites for serine and threonine residues probably are either not utilized or are, in fact, phosphorylated but of no consequence to either class I MHC antigen induction or antiviral action. The possibility that serine or threonine phosphorylations play a role in receptor activity cannot be excluded by our results, however. Potential phosphorylation sites exist in that region of the internal domain closest to the transmembrane domain. These sites were unaltered by mutagenesis and could be important in receptor internalization.

A significant observation made in this study is that protection of cells by Hu-IFN- γ against EMCV and VSV infection clearly occurs by different mechanisms, consistent with earlier reports (27, 37–39). VSV protection, in response to Hu-IFN- γ , was never seen in CHO cells containing only the Hu-IFN- γ R and human chromosome 21q. This result strongly suggests that another accessory factor may be necessary for activation of the mechanisms that provide CHO cells with protection against VSV. This second accessory factor may be sufficient for anti-VSV activity, or, alternatively, the second factor may also require the chromosome 21 factor. Correlation of intracellular domain structure with VSV resistance is contingent upon identification of the chromosome encoding this factor.

The internal deletions and truncations together define a cluster of five amino acids (YDKPH) that are conserved in the human and murine IFN- γ R and are essential for MHC antigen induction and antiviral activity against EMCV (Fig. 5). Of these amino acids, His⁴⁶¹ and Tyr⁴⁵⁷ were found necessary for activity; and a recent abstract (40) reported that Asp⁴⁵⁸ is also essential. Consistent with these results, Aguet (41) has reported that microinjection of an antibody against the carboxyl-terminal region of the Hu-IFN- γ R abolished its activity. At the present time, a most intriguing question concerns the nature of the interactions occurring at this site. Possibly, this region of the receptor is required for a phosphorylation event at Tyr⁴⁵⁷, although no tyrosine phosphor-

ylation has yet been reported on the Hu-IFN- γ R. Mao *et al.* (35) and Khurana Hershey *et al.* (36) reported only Ser/Thr phosphorylation during metabolism of the Hu-IFN- γ R. The intracellular domain may interact with cytoplasmic accessory factors yet to be defined; alternatively, the chromosome 21 accessory factor itself may bind here but not with the species specificity characteristic of its interaction with the extracellular domain because the mouse and human intracellular domains can be exchanged with full retention of function (14). Finally, it is possible that this region is required for receptor-receptor interactions (for example, dimerization). In any case, the data presented in this report show that the region defined near the carboxyl-terminal end of the Hu-IFN- γ R intracellular domain (YDKPH) plays a central role in signal transduction.

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