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Ligand-induced IFNγ receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91)

Andrew C.Greenlund, Michael A.Farrar, Beth L.Viviano and Robert D.Schreiber[1]

Department of Pathology, Washington University School of Medicine, St Louis, MO 63110, USA
[1]Corresponding author
The first two authors contributed equally to the results presented here

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Herein we report that interferon-γ (IFNγ) induces the rapid and reversible tyrosine phosphorylation of the IFNγ receptor. Using a panel of receptor intracellular domain mutants, we show that a membrane-proximal LPKS sequence (residues 266–269) is required for ligand-induced tyrosine kinase activation and/or kinase–receptor association and biological responsiveness, and a functionally critical membrane-distal tyrosine residue (Y440) is a target of the activated enzyme. The biological significance of Y440 phosphorylation was demonstrated by showing that a receptor-derived nonapeptide corresponding to receptor residues 436–444 and containing phosphorylated Y440 bound specifically to p91, blocked p91 phosphorylation and inhibited the generation of an active p91-containing transcription factor complex. In contrast, nonphosphorylated wild-type, phosphorylated mutant, or phosphorylated irrelevant peptides did not. Moreover, the phosphorylated Y440-containing peptide did not interact with a related but distinct latent transcription factor (p113) which is activatible by IFNα but not IFNγ. These results thus document the specific and inducible association of p91 with the phosphorylated IFNγ receptor and thereby elucidate the mechanism by which ligand couples the IFNγ receptor to its signal transduction system.

Key words: interferon/receptor/signal transduction/transcription factor/tyrosine phosphorylation

Introduction

Interferon-γ (IFNγ) is a cytokine produced by T cells and natural killer cells that plays important roles in promoting host defense and immunopathological processes (reviewed by Farrar and Schreiber, 1993). IFNγ exerts its pleiotropic effects on cells through an interaction with a specific high affinity receptor expressed at the cell surface (Farrar and Schreiber, 1993). Functionally active IFNγ receptors are composed of two distinct, species-specific polypeptides. A 90 kDa α chain (Aguet *et al.*, 1988) encoded by a gene on human chromosome 6 (Pfizenmaier *et al.*, 1988) and murine chromosome 10 (Mariano *et al.*, 1987) is both necessary and sufficient for ligand binding and processing and necessary, but not sufficient, for biological response induction. A second, recently cloned, polypeptide denoted the IFNγ receptor β chain (Hemmi *et al.*, 1994) or AF-1 (Soh *et al.*, 1994) encoded by a gene on human chromosome 21 (Jung *et al.*, 1987) or murine chromosome 16 (Hibino *et al.*, 1991) is needed exclusively for development of functional responses in cells. Although the function of the receptor β chain remains unclear, the structure–function relationships that exist within the receptor α chain have been the focus of recent studies (Farrar *et al.*, 1991, 1992; Cook *et al.*, 1992). These analyses, carried out by expressing wild-type or mutant human IFNγ receptor α chains in murine cell lines that also contain human chromosome 21, have identified two topographically distinct, functionally important regions within the receptor α chain's intracellular domain. The first is comprised of 48 amino acids, proximal to the receptor's transmembrane domain (amino acids 256–303) and contains elements required for both receptor-mediated ligand internalization and biological response induction (Farrar *et al.*, 1991). The second region is located near the receptor's carboxyl-terminus and contains three closely spaced amino acids, Y440, D441 and H444, which are required exclusively for biological responsiveness (Farrar *et al.*, 1992).

The increased understanding of the structure and function of the IFNγ receptor has coincided with a growth in understanding of the molecular events that underlie IFNγ-dependent signal transduction. Recently, IFNγ was shown to induce in cells the phosphorylation and activation of a latent Src homology 2 (SH2) domain-containing cytoplasmic transcription factor, named p91 (Fu, 1992; Schindler *et al.*, 1992a,b; Shuai *et al.*, 1992). Activation of p91 results in the assembly of a p91-containing multimolecular transcription factor complex which translocates to the nucleus and binds to specific sequences in the promoters of IFNγ-inducible genes, thereby initiating gene transcription (Decker *et al.*, 1991; Shuai *et al.*, 1992; Igarashi *et al.*, 1993b). Although these observations have substantially enhanced the understanding of IFNγ signal transduction, they have not yet defined the molecular mechanisms coupling the IFNγ receptor to p91 activation. Based on our previous finding that the highly conservative substitution of phenylalanine for tyrosine at position 440 in the IFNγ receptor α chain results in a functionally inactive receptor (Farrar *et al.*, 1992), we investigated whether IFNγ effected the tyrosine phosphorylation of its own receptor and thereby provided a ligand-dependent mechanism of p91–IFNγ receptor interaction.

In this report, we demonstrate that IFNγ induces the rapid and reversible tyrosine phosphorylation of the IFNγ receptor α chain. Moreover, we show that (i) a specific tetrapeptide sequence within the functionally important membrane-proximal region of the receptor is required for IFNγ receptor tyrosine phosphorylation, and (ii) the functionally important Y440 residue near the receptor's carboxyl-terminus is a target for the IFNγ-induced tyrosine kinase activity. The physiologic relevance of Y440 phosphorylation was demonstrated using IFNγ receptor-derived peptides and their

phosphorylated derivatives. In this report, we show the heretofore unrecognized specific interaction of p91 with a phosphorylated receptor-derived Y440-containing sequence. Our results thus define the roles played by each topographically distinct region of the IFNγ receptor α chain's intracellular domain and document the physiologic significance of ligand-induced IFNγ receptor α chain phosphorylation.

Results

The IFNγ receptor α chain is phosphorylated on tyrosine residues in a ligand-dependent manner

Based on our previous observation that Y440 was obligatorily required for functional activity of the IFNγ receptor, we asked whether IFNγ induced tyrosine phosphorylation of its own receptor. In the absence of ligand, no tyrosine phosphorylated IFNγ receptor α chain was detected in human Colo-205 cells by Western blot analysis (Figure 1, lane 1). Following addition of IFNγ, a rapid and reversible tyrosine phosphorylation of the receptor α chain was observed. Receptor α chain phosphorylation was maximal within 15 s (Figure 1, lane 2), remained at maximal levels for 5 min (lanes 3 and 4), was significantly reduced at 15 min (lane 5) and returned to background levels by 30 min (lane 6). The amounts of receptor loaded in each lane were equivalent as demonstrated by subjecting 20% of each solubilized immunoprecipitate to Western blot analysis using the GIR-94 anti-IFNγ receptor α chain mAb (data not shown). Similar results were obtained when IFNγ receptor α chains were immunoprecipitated from lysates of IFNγ-treated cells with antiphosphotyrosine mAb (4G10) and Western blot analysis performed with the IFNγ receptor α-chain-specific GIR-94 mAb (data not shown). Ligand-dependent tyrosine phosphorylation of the IFNγ receptor α chain was inhibited by pretreatment of the cells with herbimycin A (1 μM) (data not shown). This treatment also blocked IFNγ-dependent MHC class II induction in these cells.

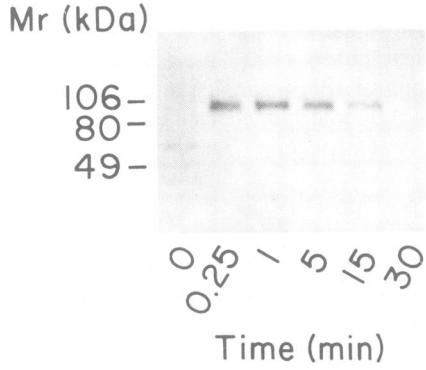

Fig. 1. Kinetics of IFNγ receptor α chain tyrosine phosphorylation. Colo-205 (1×10^8 cells/ml) were incubated in the absence of rHuIFNγ (lane 1), or presence of rHuIFNγ (10 000 IRU/ml) for 0.25 min (lane 2), 1 min (lane 3), 5 min (lane 4), 15 min (lane 5) or 30 min (lane 6) at 37°C. Cells were lysed and the IFNγ receptor α chain immunoprecipitated using the mAb GIR-94 and protein G–Sepharose. Immunoprecipitates were washed with buffer containing 1.5% NP-40, 1% SDS and 0.5% deoxycholate, subjected to SDS–PAGE and Western blotted using the biotinylated mAb 4G10 (anti-phosphotyrosine) and streptavidin–horseradish peroxidase as described in Materials and methods.

To characterize the tyrosine phosphorylation of the IFNγ receptor α chain further, we performed concomitant dose–response phosphorylation and MHC class II induction analyses. IFNγ receptor tyrosine phosphorylation was detected when Colo-205 cells (10^8 cells/ml) were treated for 5 min with 100 IRU/ml of IFNγ and reached maximal levels at IFNγ doses of 10,000 IRU/ml (Figure 2A). A similar profile was obtained when Colo-205 cells were exposed to identical doses of IFNγ for 5 min, washed, placed back in culture for 48 h and then analyzed for MHC class II expression (Figure 2B). Thus the amount of IFNγ required to induce IFNγ receptor tyrosine phosphorylation parallels that required to induce biological responses.

Ligand-dependent tyrosine phosphorylation of the IFNγ receptor α chain requires the presence of the species-specific receptor β chain

Cellular responsiveness to IFNγ requires the presence of both species matched receptor α and β polypeptide chains (Farrar and Schreiber, 1993). To determine whether ligand-induced IFNγ receptor α chain tyrosine phosphorylation showed a similar requirement, we utilized a Colo-205 derivative, designated M-Colo.22, that stably expresses the murine IFNγ receptor α chain (Hershey *et al.*, 1990). On these cells the transfected murine receptor α chain binds and processes

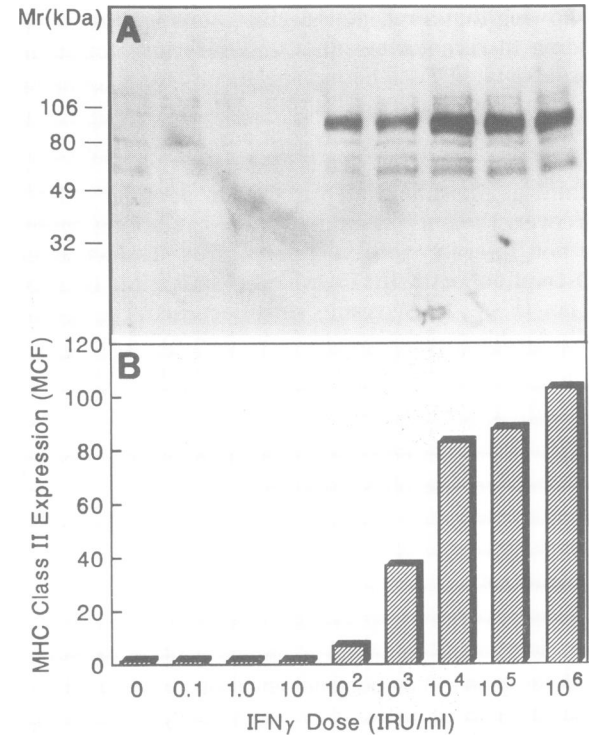

Fig. 2. Dose–response of IFNγ receptor α chain tyrosine phosphorylation and class II induction. (**A**) Colo-205 cells (1×10^8 cells/ml) were incubated for 5 min at 37°C with the indicated concentrations of rHuIFNγ. Cells were lysed and the IFNγ receptor α chain immunoprecipitated using the mAb GIR-94. Immunoprecipitates were subjected to Western blot analysis using biotinylated mAb 4G10 as in Figure 1. (**B**) Colo-205 cells (1×10^8 cells/ml) were incubated for 5 min at 37°C with the indicated concentrations of rHuIFNγ, washed, resuspended in 10 ml medium and incubated for an additional 48 h in the absence of rHuIFNγ. MHC class II expression was quantified by flow cytometric analysis as described in Materials and methods.

murine IFNγ in a normal manner but is unable to support biological responses to murine IFNγ because the cells lack the murine IFNγ receptor β chain. Treatment of M-Colo.22 with human IFNγ resulted in tyrosine phosphorylation of the endogenous human IFNγ receptor α chain (Figure 3A) but not the transfected murine receptor α chain (Figure 3B). Stimulation of cells with murine IFNγ did not result in tyrosine phosphorylation of either the human or murine α chains (Figure 3A and B, respectively). The cell line expressed comparable levels of both the human and murine receptor α chains as documented elsewhere by radioligand binding analysis (Hershey *et al.*, 1990) and herein by immunoprecipitation and Western blot analysis (Figure 3C and D, respectively). These results demonstrate that the IFNγ receptor β chain is required for the ligand-induced tyrosine phosphorylation of the IFNγ receptor α chain.

The functionally critical Y440 residue in the carboxyl-terminal region of the IFNγ receptor α chain is a target for tyrosine phosphorylation but is not required for tyrosine kinase activation

Having established the general characteristics of IFNγ-induced IFNγ receptor α chain tyrosine phosphorylation, we examined whether the functionally critical Y440 was a target for the IFNγ activatible tyrosine kinase. Due to the limited expression of IFNγ receptor α chains on cell surfaces, direct phosphoamino acid analysis of phosphorylated receptor was not possible. Therefore as an alternative approach we analyzed ligand-dependent tyrosine phosphorylation of receptor α chain mutants that either contained Y440 as the *only* intracellular domain tyrosine or specifically lacked *only* this unique functionally important tyrosine. For these studies we generated families of murine fibroblasts (SCC16-5 and WA-17) containing human chromosome 21 that stably expressed (i) the wild-type human IFNγ receptor α chain that contained all five intracellular domain tyrosine residues, (ii) an α chain mutant that contained only Y440 as the sole intracellular domain tyrosine residues (4XYF) produced by substituting phenylalanine residues for the other four intracellular domain tyrosines, or (iii) a human receptor α chain point mutant that lacked only Y440 by replacing this residue with phenylalanine (YF440). The cell lines that expressed wild-type receptor α chain or the 4XYF mutant responded comparably to human IFNγ (34 and 33 mean channel increases in MHC class I expression, respectively). In contrast, the cell line expressing the YF440 mutant was unresponsive to human IFNγ (0 mean channel increase). All three cell lines responded to their endogenous murine IFNγ indicating that there was no general defect in the MHC class I induction pathway. Furthermore, all cell lines responded to human IFNα_2, thus verifying that they contained human chromosome 21 (data not shown).

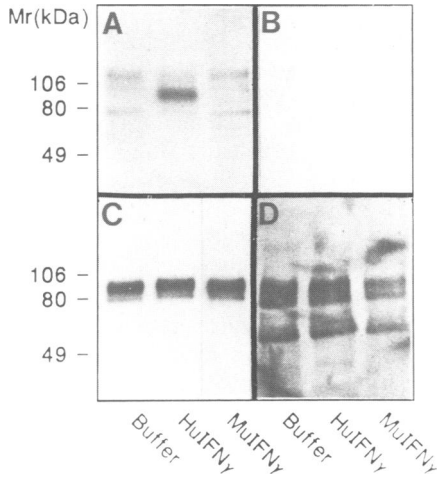

Fig. 3. IFNγ receptor α chain tyrosine phosphorylation requires the presence of the species-specific receptor β chain. M-Colo.22 cells (Colo-205 cells expressing the murine IFNγ receptor α chain) were incubated with buffer (lanes 1 and 4), rHuIFNγ (10 000 IRU/ml) (lanes 2 and 5), or rMuIFNγ (10 000 IRU/ml) (lanes 3 and 6) for 5 min at 37°C. Cells were lysed and human IFNγ receptor α chain (**A** and **C**) or murine IFNγ receptor α chain (**B** and **D**) immunoprecipitated using the mAb GIR-94 (anti-human IFNγ receptor α chain) or GR-20 (anti-murine IFNγ receptor α chain), respectively. Immunoprecipitates were washed, and subjected to Western blot analysis using biotinylated 4G10 (A and B), GIR-94 (C), or polyvalent goat anti-murine IFNγ receptor α chain serum (D) as in Figure 1.

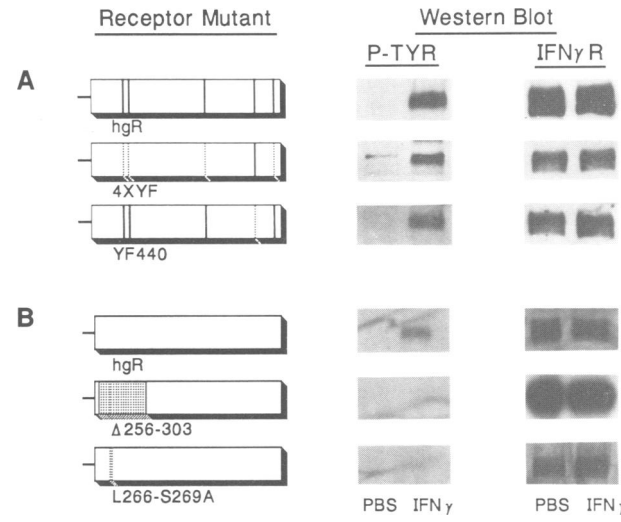

Fig. 4. IFNγ-dependent IFNγ receptor α chain tyrosine phosphorylation occurs on Y440 and other tyrosines and is dependent on distinct regions of the IFNγ receptor α chain intracellular domain. (**A**) Demonstration that Y440 is phosphorylated in a ligand-dependent manner. The wild-type human IFNγ receptor α chain containing all five intracellular tyrosine residues (hgR, line 1), a biologically active human IFNγ receptor α chain containing only a single intracellular domain tyrosine at position 440 generated by introducing phenylalanine substitutions for the other four intracellular domain tyrosines at positions 287, 294, 380 and 462 (4XYF, line 2), and a biologically inactive receptor lacking only tyrosine 440 produced by introducing a phenylalanine substitution for Y440 (YF440, line 3) were expressed in murine fibroblast cell lines containing human chromosome 21. Substitutions are identified as dotted lines, wild-type tyrosines appear as solid lines. Cells (1×10^8 cells/ml) were incubated in the presence or absence of rHuIFNγ (10 000 IRU/ml) for 30 s at 37°C and lysed, and Western blot analyses were performed on immunoprecipitated receptor α chain using the mAbs 4G10 (center column) or GIR-94 (right column) and streptavidin–horseradish peroxidase as in Figure 1. (**B**) The membrane-proximal tetrapeptide sequence, LPKS, is required for IFNγ receptor α chain tyrosine phosphorylation. SCC16-5 cells (1×10^8) stably expressing either full-length human IFNγ receptor α chain (line 1), a biologically inactive deletion mutant Δ256–303 lacking the membrane-proximal 48 amino acids (line 2), or the biologically inactive mutant L266-S269A in which the sequence LPKS was replaced by AAAA (line 3) were stimulated with rHuIFNγ (10 000 U/ml) for 5 min at 37°C and lysed. Immunoprecipitated receptors were analyzed by Western blot analysis using the mAbs 4G10 (center column) or GIR-94 (right column) and streptavidin–horseradish peroxidase as described in Figure 1.

The three transfected cell lines were then tested for their capacity to tyrosine phosphorylate the human IFNγ receptor α chain in response to human IFNγ. The cell line expressing the wild-type human IFNγ receptor α chain (hgR) that contained all five intracellular domain tyrosines showed α chain tyrosine phosphorylation in a ligand-dependent manner (Figure 4A, line 1). This result demonstrated that IFNγ-induced receptor tyrosine phosphorylation occurred in hybrid murine cells as well as natural human cells. Stimulation with human IFNγ of cells that expressed the biologically active 4XYF mutant containing only the single intracellular domain tyrosine residue at position 440 resulted in tyrosine phosphorylation of the mutant human IFNγ receptor α chain (Figure 4A, line 2) although at lower levels than those observed for the wild-type human receptor α chain. Since the 4XYF mutant contains only a single intracellular domain tyrosine residue at position 440 and since immunoprecipitation and antiphosphotyrosine Western blotting are specific, this result demonstrates that the functionally critical tyrosine residue at position 440 is indeed phosphorylated in cells following stimulation with ligand. Stimulation of cells expressing the biologically inactive YF440 mutant lacking only the single functionally important Y440 residue also effected receptor α chain tyrosine phosphorylation (Figure 4A, line 3) at levels comparable to that observed with the wild-type receptor. These results demonstrate that IFNγ effects the phosphorylation of Y440 and (probably several) other tyrosine residues within the intracellular domain of the IFNγ receptor α chain. In addition, they show that whereas the functionally critical Y440 residue is a target for the active tyrosine kinase it is not required for tyrosine kinase activation.

The kinetics of 4XYF phosphorylation were distinct from those of the wild-type receptor α chain. Although both 4XYF and wild-type receptor were maximally tyrosine phosphorylated 15–30 s after IFNγ addition to the cells, levels of tyrosine phosphorylated 4XYF became almost undetectable 5 min after IFNγ stimulation while phosphorylated wild-type receptor α chain could be readily detected up to 15 min after cell stimulation. Thus phosphorylation of Y440 is even more transient than that of the other tyrosine residues in the IFNγ receptor α chain.

An LPKS sequence within the functionally critical, membrane-proximal region of the IFNγ receptor α chain is required for α chain tyrosine phosphorylation and development of biological responses

The observations made thus far showed that although Y440 was a substrate for the IFNγ-inducible tyrosine kinase and obligatorily required for development of biological responses, it was not involved in kinase activation. We therefore asked whether the other functionally important intracellular domain region of the IFNγ receptor α chain, located near the membrane [residues 256–303 (Farrar *et al.*, 1991)], played a role in tyrosine kinase activation. To explore this possibility, we generated a panel of murine fibroblasts (SCC16-5) containing human chromosome 21 that expressed different human IFNγ receptor α chain derivatives with mutations within the membrane-proximal region. SCC16-5 cells expressing wild-type human IFNγ receptor α chain responded to human IFNγ (56 mean channel increase in MHC class I expression) and showed α chain tyrosine phosphorylation (Figure 4B, line 1). In contrast, deletion of the entire 48 amino acid membrane-proximal region (Δ256–303) resulted in an α chain that could neither support its own tyrosine phosphorylation (Figure 4B, line 2) nor effect the development of biological responses in cells (0 mean channel increase in MHC class I expression) upon exposure to human IFNγ (Farrar *et al.*, 1991). Thus, the membrane-proximal region is obligatorily required for receptor α chain tyrosine phosphorylation as well as biological response induction.

Table I. Mutation of L266-S269 in the human IFNγ receptor α chain abolishes biological responsiveness to rHuIFNγ

Mutant	MHC class I induction (mean channel shift)	
	Human IFNγ	Murine IFNγ
Human wild-type	42	48
256–260	42	40
261–265	47	43
266–269	**0**	**30**
270–271	49	55
272–276	47	46
277–281	46	45
282–286	38	37
287–292	52	54
293–298	58	57
299–303	39	38

Mutants were prepared based on the sequence of residues 256–303 (INPLKEKSIILPKSLISVVRSATLETKPESKYVSLITSYQPFSLEKE-V). Mutant nomenclature indicates the residues substituted by alanine. Mutant receptors were stably expressed in WA-17 and were tested for IFNγ responsiveness by monitoring MHC class I induction as described in Materials and methods.

To define the specific amino acids within the membrane-proximal region that were required for IFNγ-dependent receptor tyrosine phosphorylation and biological response induction, we performed an alanine scan of the receptor's membrane-proximal region. Human IFNγ receptor α chain mutants were stably expressed in WA-17 and the resulting cell lines tested for interferon-dependent enhancement of MHC class I expression. All cell lines responded to homologous murine IFNγ and to human IFNα$_2$. Only one mutant (L266-S269A) containing alanine substitutions for an LKPS sequence (residues 266–269) was identified that was deficient in IFNγ responsiveness (Table I). All other membrane-proximal domain mutants were functional in WA-17 and effected 38–52 mean channel increases in MHC class I protein expression. These results indicated that the only portion of the membrane-proximal region of the IFNγ receptor α chain absolutely required for induction of biological responses is a tetrapeptide sequence, LPKS, spanning residues 266–269.

The LPKS deletion mutant (L266-S269A) was tested for IFNγ-dependent IFNγ receptor α chain tyrosine phosphorylation. No phosphorylation was observed (Figure 4B, line 3). Taken together, these results show that the LPKS sequence contained within the membrane-proximal region of the IFNγ receptor α chain is required for both ligand-dependent receptor tyrosine phosphorylation and biological response induction and suggest that this region

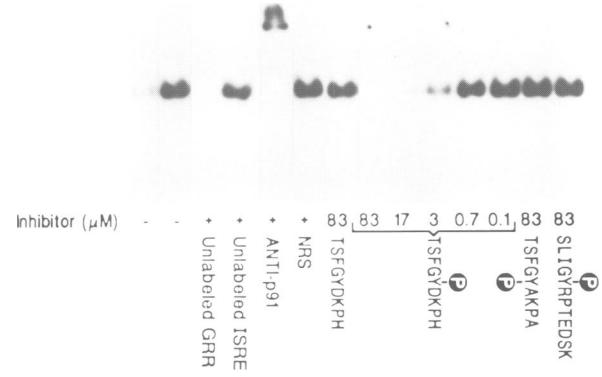

Fig. 5. A tyrosine phosphorylated IFNγ receptor α chain-derived peptide inhibits formation of a p91-containing, DNA binding complex. Colo-205 homogenates were preincubated either in the absence (lanes 1–6) or presence (lanes 7–14) of the IFNγ receptor α chain-derived peptides. Homogenates were then incubated in the presence or absence of rHuIFNγ. Extracts were prepared and the supernatant assayed for p91 activation by EMSA utilizing a ^{32}P-labeled oligonucleotide probe corresponding to the GRR of FcγRI. The following reaction conditions were: minus rHuIFNγ (lane 1), plus rHuIFNγ (lanes 2–14), addition of unlabeled GRR oligonucleotide (lane 3), addition of unlabeled ISRE of ISG15 oligonucleotide (lane 4), addition of rabbit anti-human p91 serum (lane 5) or normal rabbit serum (lane 6), preincubation with 83 μM nonphosphorylated 436–4444 peptide TSFGYDKPH (lane 7), preincubation with tyrosine phosphorylated 436–444 peptide TSFG-YPO_4-DKPH at concentrations of 83, 17, 3, 0.7, 0.1 μM (lanes 8–12, respectively), preincubation with 83 μM tyrosine phosphorylated, alanine substituted 436–444 mutant peptide TSFG-YPO_4-AKPA (lane 13), and preincubation with 83 μM tyrosine phosphorylated 456–469 irrelevant peptide SLIG-YPO_4-RPTEDSK (lane 14).

is important either for tyrosine kinase activation or kinase–receptor association.

Phosphorylation of Y440 is required for IFNγ receptor signal transduction

Having established the requirements and specificity of IFNγ-induced tyrosine phosphorylation of the functionally critical Y440 residue, we sought to determine the biological significance of this event. Due to the transient nature of Y440 phosphorylation, we explored the biological relevance of receptor tyrosine phosphorylation using phosphopeptides based on the primary sequence of the IFNγ receptor α chain. For this study we took advantage of the finding that the latent IFN-inducible transcription factor, p91, can be activated by IFNγ in a cell-free system (Igarashi *et al.*, 1993a). Activation of p91 was monitored functionally using an electrophoretic mobility shift assay (EMSA) that employed a ^{32}P-labeled 18 bp probe derived from the FcγRI gene promoter (GRR) (Pearse *et al.*, 1993) and biochemically by monitoring p91 tyrosine phosphorylation. In the absence of ligand, little or no activated p91 was observed in the EMSA (Figure 5, lane 1). In contrast, addition of human IFNγ to the homogenate resulted in the generation of a prominent retarded band (lane 2). The specificity of the activated DNA binding complex was confirmed by demonstrating that unlabeled GRR probe inhibited the formation of the band (lane 3) while an unlabeled oligonucleotide corresponding to the interferon-stimulated responsive element (ISRE) of ISG15 (Levy *et al.*, 1988) did not (lane 4). The presence of p91 in the gel shift complex was demonstrated using a rabbit antiserum specific for the carboxyl-terminal portion of human p91 (Schindler *et al.*, 1992a). Whereas anti-p91 serum effected a supershift of the transcription factor complex when added to the reaction mixture following addition of labeled probe (lane 5), normal rabbit serum was without effect (lane 6). These results thus validate the EMSA used in our laboratory and establish that p91 can be activated by ligand in homogenates of Colo-205.

To explore the role of Y440 in p91 activation, we generated a series of 9 and 12 amino acid peptides based on sequences from the IFNγ receptor α chain intracellular domain. Pretreatment of Colo-205 homogenates with a peptide corresponding to residues 436–444 of the human IFNγ receptor α chain (TSFGYDKPH) had little or no effect on p91 activation, even when used at a final concentration of 83 μM (Figure 5, lane 7). In contrast, pretreatment with a phosphotyrosine-containing nonapeptide with the same sequence blocked the activation of p91 in a dose-dependent manner (lanes 8–12). Formation of an activated p91-labeled probe complex was completely inhibited at phosphopeptide inputs of 83 and 15 μM and 61% inhibited at phosphopeptide concentrations of 3 μM. Lower concentrations of the phosphorylated nonapeptide (0.7 and 0.1 μM) were not inhibitory. Identical results were obtained with the 12 amino acid versions of the peptides that contained an additional three amino acids at the carboxyl-terminus (VLV). The specificity of the inhibition was confirmed using two additional phosphopeptides. No inhibition was noted when a mutated 436–444 phosphopeptide was used in which the functionally critical D441 and H444 residues (Farrar *et al.*, 1992) were changed to alanine (lane 13). In addition, no inhibition was observed when a phosphorylated 12 amino acid peptide was used that was based on an adjacent tyrosine-containing IFNγ receptor α chain intracellular domain sequence (residues 458–469) that is not functionally important (Farrar *et al.*, 1992) (lane 14).

To confirm that the tyrosine phosphorylated 436–444 peptide blocked p91 activation, the ligand-dependent phosphorylation of p91 was monitored in a cell-free system. No phosphorylated p91 was detected in unstimulated Colo-205 homogenates (Figure 6A, lane 1). However, following addition of IFNγ, tyrosine phosphorylation of p91 was induced (lane 2). Tyrosine phosphorylation of p91 was not affected if nonphosphorylated 436–444 (lane 3), mutated phosphorylated 436–444 containing the DA441 and HA444 substitutions (lane 5) or the irrelevant 458–469 phosphorylated peptide (lane 6) was added to the homogenate before IFNγ stimulation. In contrast, preincubation of the homogenate with the phosphorylated 436–444 peptide inhibited ligand-induced p91 tyrosine phosphorylation (lane 4). In this particular experiment, the phosphorylated 436–444 peptide (140 μM) inhibited ligand-induced p91 phosphorylation by 80% as determined by densitometry. In subsequent experiments in which only the phosphorylated 436–444 peptide was used as an inhibitor at concentrations of 147 μM, complete inhibition of p91 phosphorylation was observed (data not shown). The apparent difference in peptide concentration needed to inhibit p91 phosphorylation as compared with assembly of the active p91-containing transcription factor complex can largely be explained by the fact that the concentration of lysate in the former assay is six times higher than that used in the EMSA. Equivalent

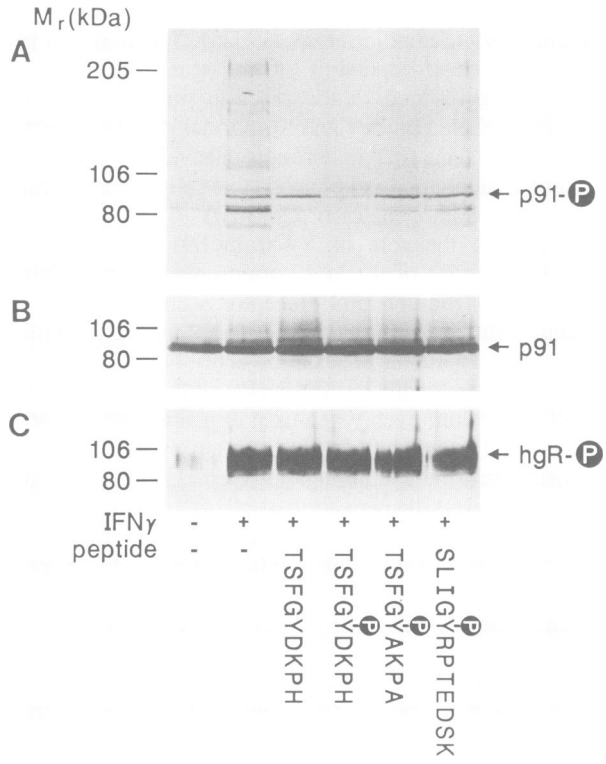

Fig. 6. The tyrosine phosphorylated IFNγ receptor α chain-derived peptide inhibits IFNγ-induced p91 activation. Colo-205 homogenates were incubated with: no peptide (lanes 1 and 2), nonphosphorylated 436–444 peptide TSPGYDKPH (lane 3), tyrosine phosphorylated 436–444 peptide TSFG-YPO_4-DKPH (lane 4), tyrosine phosphorylated alanine substituted 436–444 peptide TSFG-YPO_4-AKPA (lane 5), or tyrosine phosphorylated irrelevant 458–469 peptide SLIG-YPO_4-RPTEDSK (lane 6) at a concentration of 140 μM for 1 h prior to incubation with rHuIFNγ (lanes 2–6). After rHuIFNγ stimulation, homogenates were solubilized and cleared by centrifugation. p91 was immunoprecipitated using a rabbit anti-human p91 immune serum and protein A–agarose. After clearing the lysate of p91, the IFNγ receptor α chain was immunoprecipitated using GIR-94 and protein A–agarose. Immunoprecipitates were washed, subjected to SDS–PAGE, and the tyrosine phosphorylation state of p91 assessed by Western blotting using the anti-phosphotyrosine mAb RC20 (**A**). The nitrocellulose membranes were stripped after anti-phosphotyrosine blotting and blotted for p91 using a rabbit anti-p91 immune serum and peroxidase conjugated goat anti-rabbit IgG (**B**). The tyrosine phosphorylation state of the IFNγ receptor α chain was also assessed using RC-20 (**C**).

amounts of p91 were immunoprecipitated in the absence or presence of the various peptides (Figure 6B). Moreover, none of the peptides inhibited IFNγ receptor α chain phosphorylation (Figure 6C). Thus, the phosphorylated 436–444 peptide specifically inhibits p91 activation and not IFNγ receptor phosphorylation. These results thus show that the Y440-containing phosphopeptide specifically blocks the activation of a recognized effector molecule in the IFNγ signaling pathway (p91) and suggest that phosphorylation of Y440 is a critical event in IFNγ signal transduction.

Demonstration of a specific interaction of p91 with the phosphorylated YDKPH sequence

One explanation for the observations described above was that p91 activation occurred as a result of the binding of p91 to the IFNγ receptor α chain and that this association was made possible by the ligand-induced phosphorylation of

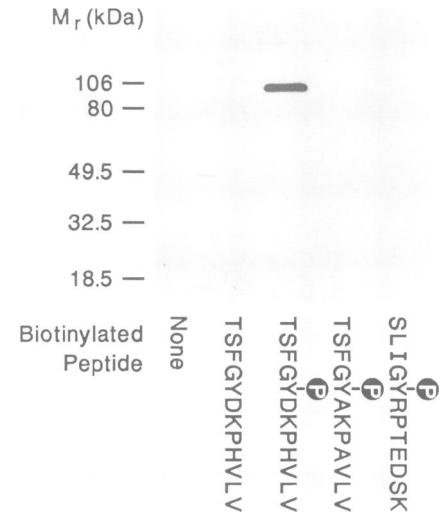

Fig. 7. Coprecipitation of p91 with a tyrosine phosphorylated IFNγ receptor γ chain-derived peptide containing Y440. Solublized Colo-205 homogenates were incubated without peptide (lane 1) or with the following biotinylated IFNγ receptor α chain-derived peptides: biotinylated 436–447 peptide TSFGYDKPHVLV (lane 2), biotinylated phosphorylated 436–447 peptide TSFG-YPO_4-DKPHVLV (lane 3), biotinylated phosphorylated alanine substituted 436–447 TSFG-YPO_4-AKPAVLV (lane 4), and biotinylated phosphorylated irrelevant 458–469 peptide SLIG-YPO_4-RPTEDSK (lane 5). Biotinylated peptides were then precipitated with streptavidin–Sepharose, washed with buffer containing 0.5% NP-40 and subjected to Western blot analysis using a rabbit anti-human p91 immune serum and peroxidase conjugated goat anti-rabbit IgG.

Y440. We therefore explored whether p91 interacted with the phosphorylated YDKPH containing receptor α chain-derived peptide. Derivatives of the four peptides described above containing biotinylated amino-terminal amino acids were incubated with Colo-205 homogenates and precipitated with streptavidin–agarose. The presence of p91 in the precipitates was assessed by SDS–PAGE and Western blot analysis using p91-specific antiserum. No p91 was precipitated either in the absence of peptide (Figure 7, lane 1) or in the presence of nonphosphorylated, biotinylated wild-type 436–447 peptide (TSFGYDKPHVLV, lane 2). In contrast, p91 was clearly evident in precipitates formed with phosphorylated, biotinylated 436–447 peptide (lane 3). No p91 was precipitated using either the phosphorylated, biotinylated 436–447 peptide mutant (lane 4) or the phosphorylated, biotinylated irrelevant 458–469 peptide (lane 5). Thus the specificity of the interaction between p91 and the receptor-derived phosphopeptide observed using this coprecipitation approach corresponded to that observed using the EMSA functional and p91 phosphorylation assays.

Finally, the specificity that the phosphorylated IFNγ receptor α chain sequence showed for p91 versus other cytosolic transcription factors was assessed. Although the biotinylated phosphorylated 436–447 peptide precipitated p91 from Colo-205 homogenates (Figure 8, lane 1), it did not precipitate p113, a transcription factor specifically induced by IFNα but not IFNγ (lane 2) (Shuai *et al.*, 1992). To control for the possibility that Colo-205 might express vastly different quantities of the two transcription factors, p91 and p113 were directly immunoprecipitated from homogenates using the respective specific antisera and quantified by Western blotting. Equivalent amounts of each

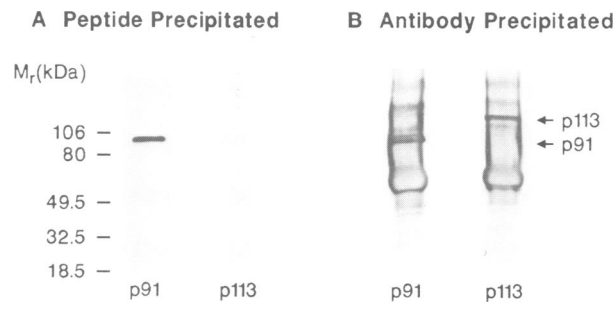

Fig. 8. The tyrosine phosphorylated IFNγ receptor α chain-derived peptide containing Y440 interacts with p91 but not p113. (**A**) Solubilized Colo-205 homogenates were incubated with biotinylated phosphorylated 436–444 peptide (biotin-TSFG-YPO_4-DKPHVLV) (lanes 1 and 2). The biotinylated peptide was precipitated using streptavidin–Sepharose and the precipitate washed with buffer containing 0.5% NP-40. Precipitates were analyzed by Western blotting using rabbit anti-p91 (lane 1) or rabbit anti-p113 (lane 2) antisera. (**B**) Colo-205 (3×10^7 cells) (lanes 3 and 4) were solubilized and cleared by centrifugation. Anti-p91 (lane 3) or anti-p113 (lane 4) serum was added. Following a 1 h incubation, antibody was precipitated by addition of protein A–agarose. Immunoprecipitates were washed and subjected to Western blot analysis using either rabbit anti-p91 (lane 3) or rabbit anti-p113 (lane 4) serum.

protein were detected (Figure 8, lanes 3 and 4). Thus, ligand-dependent phosphorylation of Y440 leads to the generation of a specific site on the IFNγ receptor α chain to which p91 can bind.

Discussion

This study shows that IFNγ induces the rapid and reversible tyrosine phosphorylation of the IFNγ receptor α chain. The factors required for ligand-dependent receptor α chain phosphorylation match those needed for development of biological responses in cells. This study also elucidates the roles played by the two functionally important intracellular domain regions of the IFNγ receptor α chain by identifying their respective actions in the phosphorylation process. The membrane-proximal region (residues 256–303) contains an LPKS sequence (residues 266–269) that is required for activation of an IFNγ-inducible tyrosine kinase and/or forms the site on the receptor to which the kinase binds. In contrast, the membrane-distal portion, which contains the functionally important Y440, D441 and H444 residues, is the physiologically relevant target of the ligand-induced tyrosine kinase. The biological relevance of IFNγ-induced IFNγ receptor phosphorylation at Y440 was demonstrated by showing that a phosphotyrosine-containing peptide corresponding to receptor α chain amino acids 436–444 interacted with the p91 transcription factor and blocked its activation. The interaction was specific and dependent upon the presence of phosphotyrosine and the other two functionally important amino acids in the YDXXH sequence. These results thus identify the molecular alterations that occur in the IFNγ receptor α chain that give rise to the ligand-induced coupling of the IFNγ receptor system to the p91 signal transduction pathway.

Although there was no pre-existing evidence for ligand-induced tyrosine phosphorylation of the IFNγ receptor, previous studies showed that IFNγ induced phosphorylation of its receptor on serine and threonine residues (Hershey *et al.*, 1990; Mao *et al.*, 1990). Three important differences exist between ligand-induced tyrosine versus serine/threonine IFNγ receptor phosphorylation. First, whereas tyrosine phosphorylation is rapid and reversible, serine/threonine phosphorylation is slow and relatively stable. Second, receptor α chain tyrosine phosphorylation shows an obligatory requirement for the presence of both species matched IFNγ receptor α and β polypeptide chains. In contrast, serine/threonine phosphorylation occurred on both active human and inactive murine IFNγ receptor α chains when the same cells were treated with human IFNγ (Hershey *et al.*, 1990). Third, a specific tyrosine residue (Y440) has been identified in the intracellular domain of the IFNγ receptor α chain that is both a target for the IFNγ-inducible tyrosine kinase and required for development of biological responses in cells. In contrast, no functionally important serine or threonine residues have yet been identified in the receptor's intracellular domain (Farrar *et al.*, 1991, 1992). Thus taken together, these results suggest that whereas ligand-induced receptor tyrosine phosphorylation is an obligate step in the IFNγ signal transduction pathway, serine/threonine phosphorylation of the receptor is not.

The data presented herein elucidate the roles played by the two functionally important regions within the intracellular domain of the IFNγ receptor α chain. The membrane-proximal region contains an LPKS sequence (residues 266–269) that is obligatorily required for induction of both IFNγ-dependent tyrosine kinase activity and cellular responses. Interestingly, this sequence bears weak homology to so-called box 1 sequences found in the membrane-proximal regions of several receptors that are thought to associate with tyrosine kinases (Murakami *et al.*, 1991; Miura *et al.*, 1993). These include components of the receptors for erythropoietin, IL-2, GM-CSF, IL-4, IL-6 and IL-7. Mutations that included the box 1 sequence of the IL-6 receptor-associated gp130 resulted in the concomitant loss of both ligand-induced gp130 tyrosine phosphorylation and proliferative responses (Murakami *et al.*, 1991). This observation has led to the hypothesis that box 1 sequences are involved in the association of tyrosine kinases with the intracellular domains of specific receptors. Although the majority of box 1 sequences contain two closely spaced proline residues in an IPXP motif, the IL-7 receptor box 1 sequence contains only a single proline (LPDH). Thus it is possible that the functionally critical LPKS sequence in the IFNγ receptor α chain is the site of association of an IFNγ activatible tyrosine kinase.

A direct interaction of the IFNγ receptor α chain with a specific tyrosine kinase has not yet been demonstrated. However, two mutant cell lines devoid of IFNγ responsiveness (U4A and γ1A) have recently been shown to be deficient in two closely related tyrosine kinases known as JAK-1 and JAK-2, respectively (Müller *et al.*, 1993; Watling *et al.*, 1993). Moreover, IFNγ has been shown to induce the rapid activation of these enzymes in normal cells. Thus it is possible that one of these kinases associates with the IFNγ receptor α chain LPKS sequence. Interestingly, the intracellular domain of the IFNγ receptor β chain contains a box 2-like sequence element (Hemmi *et al.*, 1993). Box 2 sequences also appear to be important for mediating association of tyrosine kinases with receptors (Murakami *et al.*, 1991; Miura *et al.*, 1993; Witthuhn *et al.*,

1993). It is therefore possible that the receptor β chain may also carry one of the JAK kinases. Addition of IFNγ to cells could thereby cause the association of the α and β chains of the receptor leading to the clustering and transactivation of the receptor associated tyrosine kinases. This hypothesis would explain why both the receptor α and β chains are required for ligand-dependent receptor tyrosine phosphorylation.

The membrane-distal portion of the receptor is the functionally important target of the IFNγ activatible tyrosine kinase. The unequivocal importance of tyrosine 440 within this region has been established by our previous mutagenesis work (Farrar *et al.*, 1992). The findings delineated in this communication now elucidate the specific role of this residue in receptor function. Phosphorylation of Y440 is a prerequisite for activation of p91 and generation of DNA binding activity. The event that effects IFNγ-dependent p91 activation is the interaction of p91 with the phosphorylated tyrosine-containing YDKPH sequence in the IFNγ receptor α chain. This conclusion is based on four findings. First, Y440 was phosphorylated in response to ligand in intact cells. Although Y440 was not the only tyrosine residue to become phosphorylated, it is the only one of the five tyrosines within the receptor's intracellular domain that has been identified thus far as being functionally important. Second, a peptide consisting of amino acids 436–444 (or 436–447) of the receptor α chain and containing phosphorylated tyrosine at the position corresponding to Y440 efficiently and specifically blocked ligand-dependent p91 activation in a cell-free system. Third, the same phosphopeptide that inhibited p91 activation also coprecipitated p91 in a specific manner. This interaction showed specificity both at the peptide and transcription factor levels. Nonphosphorylated 436–444 (or 436–447) peptide neither interacted with nor blocked the activation of p91. Mutation of the functionally important aspartic acid and histidine residues within this sequence ablated phosphopeptide–p91 interaction. Moreover, a phosphotyrosine-containing peptide generated from a functionally unimportant adjacent sequence in the IFNγ receptor α chain (458–469) was also inactive. Thus p91 shows specificity for the phosphorylated YDKPH sequence in the intracellular domain of the IFNγ receptor α chain. Finally, the ability of this sequence to bind selectively to p91 and not to p113, a transcription factor that is activated by IFNα but not IFNγ (Shuai *et al.*, 1992), establishes the functional specificity of the interaction. Attempts to coprecipitate p91 directly with the IFNγ receptor α chain from IFNγ-treated cells have not yet succeeded. It is likely that the transient nature of Y440 phosphorylation and the limited expression of IFNγ receptors on normal cells have hampered these experiments. Current efforts are now focused on demonstrating the direct interaction of p91 with intact, phosphorylated IFNγ receptor intracellular domain.

The region of p91 responsible for binding to the phosphorylated IFNγ receptor Y440 containing sequence is currently unknown. However, it is possible that this interaction is mediated by the SH2 domain present in p91 (Fu, 1992). A similar suggestion has recently been made to explain the interaction of p91 (or a closely related family member) with the epidermal growth factor receptor (Fu and Zhang, 1993). However, in this case the receptor sequence responsible for mediating attachment of p91 was not identified. The p91 SH2 domain has previously been subjected to a specificity analysis using a randomized library of phosphopeptides in which the three residues at positions +1 to +3 from the phosphotyrosine were altered (Songyang *et al.*, 1993). Although this approach identified binding motifs for several other SH2 containing proteins, no phosphopeptide was identified that bound to the SH2 domain of p91. The observation made herein that the p91 reactive peptide contains critical residues in the +1 and +4 positions indicates either that the p91 SH2 domain displays an unusual binding site for its phosphorylated ligand or that p91 association with the phosphorylated IFNγ receptor α chain is mediated through an intermediate adaptor protein.

The structure–function studies carried out on the IFNγ receptor α chain establish a model whereby topographically distinct regions within the intracellular domain of a receptor lacking endogenous enzymatic activity perform temporally distinct and sequential signal transduction functions. Although the intracellular domains of certain other receptors [such as the IL-2 receptor β chain (Hatakeyama *et al.*, 1989) and the granulocyte colony stimulating factor receptor (Fukunaga *et al.*, 1993)] contain functionally distinct intracellular domain regions, no data are yet available linking the actions of these regions to the development of a single biological response. The observations made using the IFNγ receptor system suggest that a tyrosine kinase associated with one intracellular domain region can effect the ligand-dependent activation of a second region, thereby coupling the receptor to its signal transduction system. This model may prove to be a general paradigm for receptors that lack endogenous tyrosine kinase activity.

Based on the observations made in this study and the past studies of several laboratories, the following model for IFNγ signal transduction can be envisioned. Upon binding to the cell surface, IFNγ, a homodimeric ligand, induces the rapid dimerization of the IFNγ receptor α chain (Fountoulakis *et al.*, 1992; Dighe *et al.*, 1993; Greenlund *et al.*, 1993). The IFNγ receptor β chain then associates with this complex bringing into close juxtaposition at least two inactive tyrosine kinases, one associated with the LPKS box 1-like sequence of the receptor α chain and the other associated with the box 2-like sequence of the receptor β chain. It is possible that these kinases may in fact be JAK-1 and JAK-2 (Müller *et al.*, 1993; Watling *et al.*, 1993). These enzymes transactivate one another and this event leads to the phosphorylation of the Y440 residue of the IFNγ receptor α chain forming either a direct binding site for latent p91 or a site for an adaptor protein to which p91 can bind. Docking of p91 at the IFNγ receptor α chain brings p91 into close proximity with the activated receptor-associated tyrosine kinase, thereby resulting in the tyrosine phosphorylation and activation of p91. Phosphorylated p91 dissociates from the receptor, associates with either another phosphorylated p91 molecule or other activated transcription factors, and translocates to the nucleus where it interacts with specific sequences in IFNγ-inducible genes, thereby initiating gene transcription. Perhaps one of the most striking features of this model is that it is comprised of signal transduction effector molecules that are shared by a number of different receptors. It will therefore be important to define how this system functions to effect the development of IFNγ-specific biological responses.

Materials and methods

Reagents and antibodies

Purified recombinant human and murine IFNγ (sp. act. = 3.8×10^7 IRU/mg and 4.7×10^6 IRU/mg, respectively) were generously provided by Genentech (South San Francisco, CA). Recombinant human IFNα_2 (sp. act. = 1.7×10^8 IRU/mg), a species-specific form of human IFNα, was generously provided by Dr Marvin Siegel (Schering-Plough, Bloomfield, NJ).

GIR-208 and GIR-94 are murine mAbs specific for distinct epitopes on the human IFNγ receptor α chain (Sheehan *et al.*, 1988). GR-20 is a rat IgG_{2a} mAb specific for the murine IFNγ receptor α chain (Basu *et al.*, 1988). 4G10, a murine IgG_{2b} anti-phosphotyrosine mAb (Drucker *et al.*, 1989) was generously provided by Dr Brian Drucker (Dana Farber Cancer Center, Boston, MA). Purified antibodies were biotinylated as previously described (Sheehan *et al.*, 1988). The mAb 11-4.1 recognizes murine H-$2K^k$ and was purified as previously described (Farrar *et al.*, 1991). Biotin-conjugated antibodies directed against nonpolymorphic regions of HLA-DR were obtained from Becton Dickinson (Mountain View, CA). Polyclonal antisera specific for the carboxyl-terminal region of human p91 [antiserum number 29130 (Schindler *et al.*, 1992a)] and p113 [antiserum number 2994 (Fu *et al.*, 1992)] were generously given by Dr Chris Schindler (Columbia University, New York, NY). Polyclonal goat antiserum specific for the murine IFNγ receptor was generously provided by Drs Judith Pace and Stephen Russell (University of Kansas, Kansas City, KA). Protein G–Sepharose was obtained from Pharmacia (Piscataway, NJ), and protein A–agarose from Sigma (St Louis, MO). Goat anti-murine Ig and anti-rat Ig Sepharose were prepared as previously described (Hershey and Schreiber, 1989). Streptavidin–phycoerythrin was purchased from Chromoprobe (Redwood, CA) and streptavidin–horseradish peroxidase from Zymed (South San Francisco, CA). RC-20, a recombinant form of the anti-phosphotyrosine mAb PY-20 directly conjugated to horseradish peroxidase, was purchased from Transduction Laboratories (Lexington, KY).

Cells and cell culture

SCC16-5, a murine fibroblast cell line that contains a single copy of human chromosome 21 (Janssen *et al.*, 1986), WA-17, a murine L cell line that contains three copies of human chromosome 21 (Raziuddin *et al.*, 1984), and Colo-205, a human adenocarcinoma cell line, were cultured as previously described (Farrar *et al.*, 1991; Greenlund *et al.*, 1993; Hershey *et al.*, 1990, respectively).

Plasmid construction

Construction of IFNγ receptor α chain deletion mutants and carboxy-terminal point mutants has been previously described (Farrar *et al.*, 1991, 1992). The membrane-proximal region and 4XYF receptor mutants were generated in a similar manner using primers based on the nucleotide sequence of the human IFNγ receptor cDNA (Aguet *et al.*, 1988). The accuracy of all PCR-generated DNA was confirmed by dideoxy sequencing (Sequenase, US Biochemical, Cleveland, OH).

DNA transfection

SCC16-5 and WA-17 were transfected using the calcium phosphate precipitation method as previously described (Farrar *et al.*, 1992). The murine IFNγ receptor α chain was transfected into Colo-205 by electroporation as described (Hershey *et al.*, 1990).

Analysis of transfected murine cells for responsiveness to human IFNγ

The ability of human IFNγ to enhance MHC class I antigen expression on transfected murine fibroblasts was examined as previously described (Farrar *et al.*, 1991). To monitor IFNγ induction of MHC class II antigens on Colo-205, 5×10^6 cells were resuspended in 50 μl of PBS containing 10% fetal calf serum (FCS) and stimulated for 5 min at 37°C with different doses of recombinant human IFNγ. Cells were washed four times to remove IFNγ and cultured in 10 ml of medium for 48 h. HLA-DR expression was quantified by flow cytometry as described by Hershey *et al.* (1990).

Determination of receptor tyrosine phosphorylation

Cells (1×10^8) were resuspended in 1 ml of PBS–10% FCS, equilibrated at 37°C, and then treated with either PBS or rHuIFNγ. The reaction was stopped by adding 4 ml of ice-cold PBS. Cells were pelleted, washed once in ice-cold PBS and then lysed in 1 ml of lysis buffer (25 mM Tris–HCl, pH 8.0, containing 1.5% NP-40, 150 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM PMSF, 5 mM iodoacetamide, 10 μg/ml each of leupeptin and aprotinin). Solubilized cells were centrifuged for 5 min at 15 000 *g* to remove cell nuclei and the supernatants were then incubated with 10 μg of GIR-94 for 30 min at 4°C. Protein G–Sepharose was added to the reaction mixture and the incubation was continued for an additional 30 min. The beads were pelleted by centrifugation (10 000 *g* for 3 min at 4°C), washed three times with lysis buffer containing 0.5% BSA and a detergent mixture consisting of 1.5% NP-40, 1% SDS and 0.5% deoxycholate and then twice with PBS containing 1 mM sodium orthovanadate. Beads were resuspended in 50 μl of 2 × Laemmli buffer containing 180 mM β-mercaptoethanol, heated to 65°C for 5 min and pelleted by centrifugation. The supernatants were removed and then 20% of each sample was used for Western blotting with GIR-94 (anti-IFNγ receptor) and 80% for Western blotting with 4G10 (anti-phosphotyrosine). Samples were subjected to SDS–PAGE using 4–15% gradient polyacrylamide gels (Bio-Rad, Richmond, CA) and fractionated proteins were transferred electrophoretically to nitrocellulose membranes (Bio-Rad). Nitrocellulose membranes were blocked for 1–18 h at 4°C in PBS containing 5% non-fat dry milk. The membranes were then washed with PBS containing 0.05% Tween-20 and incubated for 1 h with either biotinylated GIR-94 or 4G10 (1 μg/ml). The membranes were washed again and incubated for 20 min with streptavidin–horseradish peroxidase and subsequently developed by chemiluminescence using Amersham's (Arlington Heights, IL) ECL Western blotting system.

Peptide synthesis and analysis

Peptides were synthesized manually using the Fmoc strategy on a RaMPS multiple peptide synthesis system (DuPont, Wilmington, DE) (Caprino and Han, 1972). After addition of Fmoc-Tyr(PO_3Me_2)-OH (Bachem, Bubendorf, Switzerland), 35% piperidine/dimethylformamide (DMF) was used to remove the Fmoc groups. For the dodecapeptides, biotin groups were added to the amino-termini of the peptides during the solid phase synthesis. Specifically, after the last piperidine treatment to remove Fmoc groups, a 5-fold molar excess of biotin amidocaproate *N*-hydroxysuccinimide ester (Sigma) was suspended in 3 ml dimethylsulfoxide containing 1% *N*-methylmorpholine and added to the cartridge. The slurry was rocked for 2 h and washed three times in DMF and three times in methanol, and then an Isatin test was performed to monitor completion of coupling. All peptides, phosphorylated and non-phosphorylated, were cleaved and deprotected essentially as described by Kitas *et al.* (1993) with 5 ml of 12.9% bromotrimethylsilane, 11.8% thioanisol, 75% trifluoroacetic acid (TFA), and 0.8% *m*-cresol for 16 h at 4°C. Peptides were precipitated with *tert*-butyl methyl ether, redissolved in TFA and subsequently precipitated and washed five times with ether, resuspended in H_2O and lyophilized. Reversed phase HPLC was performed using a Vydac C18 column (Hesperia, CA) and a single major peak was observed for each peptide. Electrospray mass spectrometry was performed on unfractionated peptides; a single moiety was detected which displayed the appropriate molecular mass for each peptide. Amino acid composition was verified and molarity calculated using a Beckman 6300 amino acid analyzer. Identical results were obtained using either HPLC-purified or unpurified peptides in the experiments.

Electrophoretic mobility shift assay

Colo-205 cells (2×10^8) were washed in PBS, resuspended in 1 ml of reaction buffer (100 mM HEPES buffer pH 7.5 containing 20 mM $MgCl_2$, 100 mM NaCl, 200 μM ascorbic acid, 4 mM ATP, 2 mM EGTA, 1 mM PMSF and 10 μg/ml each of leupeptin and aprotinin) and disrupted in a steel Dounce until no intact cells remained. Homogenates were diluted to a protein concentration of 6.5 mg/ml in reaction buffer and 50 μl aliquots were incubated at 4°C for 1 h in either the absence or presence of 5 μl of specific peptide. Recombinant human IFNγ (1900 IRU in 5 μl) was added and the reaction mixture incubated for 5 min at 37°C. The reactions were stopped by the addition of 450 μl of stop buffer [20 mM HEPES, pH 7.4 containing 1 mM $MgCl_2$, 10 mM KCl, 20% glycerol, 500 μM dithiothreitol (DTT), 0.1% NP-40, 1 mM PMSF, and 10 μg/ml each of leupeptin and aprotinin]. Mixtures were vortexed for 5 s and nuclei pelleted by centrifugation at 16 000 *g* for 5 min. Analysis of DNA binding activity was performed essentially as described by Igarashi *et al.* (1993a) using an oligonucleotide probe based on the 3′ 18 base pairs of the gamma response region (GRR) of the FcγRI gene (Wilson and Finbloom, 1992; Pearse *et al.*, 1993). Top strand: 5′-ATGTATTTCCCAGAAA-3′; bottom strand: 5′-CTTTTCTGGGAAATA-3′. Assays were performed by mixing 5 μg of extract with 25 000 c.p.m. (~1 ng) of the double-stranded, ^{32}P-labeled GRR probe and 4 μg poly(dIdC)(dIdC) (Pharmacia) in 24 μl of binding buffer (10 mM Tris–HCl, pH 7.5, containing 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 10% glycerol) and incubated for 20 min at room temperature. The reaction mixtures were then separated by electrophoresis through a 6% polyacrylamide gel in 0.25 × TBE for 3 h at 150 V. Gels

were dried and subsequently analyzed by autoradiography and on a Molecular Dynamics Phosphoimager (Sunnyvale, CA). Supershifts were performed using antiserum to the carboxy-terminus of human p91 (Schindler *et al.*, 1992a) at a dilution of 1:100. The specificity of interactions was determined by adding 350 ng of the unlabeled GRR probe or 560 ng of the unlabeled, 41 bp, double-stranded oligonucleotide containing the ISRE of ISG15 (5′-GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCCA-C-3′) (Levy *et al.*, 1988).

Determination of p91 tyrosine phosphorylation in a cell-free system

Aliquots (540 μl, protein concentration of 40 μg/μl) of Colo-205 homogenates were incubated in the absence or presence of 140 μM peptide for 1 h at 4°C, and then treated for 5 min at 37°C with 54 μl of rHuIFNγ (380 IRU/μl). Reactions were stopped by adding 2.5 ml of lysis buffer (50 mM Tris−HCl, pH 8.0, containing 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, 750 μM DTT, 1 mM PMSF and 10 μg/ml each of leupeptin and aprotinin). Nuclei were removed by centrifugation and the supernatant was precleared with 4 μl normal rabbit serum and 60 μl protein A−agarose for 45 min at 4°C. p91 was immunoprecipitated from the cleared supernatant using 6 μl of rabbit anti-p91 serum (number 29130) and 80 μl of protein A−agarose. Subsequently, IFNγ receptor α chain was immunoprecipitated from the treated supernatant using GIR-94. Immunoprecipitates were washed four times in lysis buffer containing 7.5% glycerol, subjected to SDS−PAGE on 6% polyacrylamide gels, and Western blotted with anti-phosphotyrosine mAb RC-20, using the protocol supplied by Transduction Laboratories. After phosphotyrosine blotting, the membranes were stripped according to the ECL protocol (Amersham) and reblotted for p91 using p91-specific antiserum.

p91 precipitation using biotinylated peptides

Aliquots (500 μl; 15 μg protein/μl) of Colo-205 homogenates were solubilized in 2.5 ml stop buffer and nuclei cleared by centrifugation. The supernatant was then incubated with the various biotinylated peptides at a final concentration of 12 μM for 1.5 h at 4°C and immunoprecipitation was carried out using 175 μl of streptavidin−Sepharose (Pierce, Rockford, IL). The Sepharose was pelleted and washed five times with 20 mM HEPES buffer pH 7.4 containing 150 mM NaCl, 0.5% NP-40, 5% glycerol, 1 mM $MgCl_2$, 500 μM DTT, 1 mM PMSF, 10 μg/ml each of leupeptin and aprotinin, 1 mM iodoacetamide, 1 mM EDTA and 1 mM sodium orthovanadate, and subjected to SDS−PAGE on 4−15% gradient gels and Western blot analysis using p91- or p113-specific antisera, as described by Schindler *et al.* (1992a). To immunoprecipitate p91 and p113 directly from Colo-205, 3×10^7 cells were solubilized in 1 ml of lysis buffer (50 mM Tris−HCl, pH 8.0 containing 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, 750 μM DTT, 1 mM PMSF and 10 μg/ml each of leupeptin and aprotinin), and nuclei were removed by centrifugation. Supernatants were precleared with 3 μl normal rabbit serum and 30 μl protein A−agarose for 45 min at 4°C and then immunoprecipitation was carried out using 5 μl of rabbit anti-p91 (serum number 29130) or rabbit anti-p113 (serum number 2994) serum and protein A−agarose.

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