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The Antiviral Action of Interferon Is Potentiated by Removal of the Conserved IRTAM Domain of the IFNAR1 Chain of the Interferon α/β Receptor: Effects on JAK-STAT Activation and Receptor Down-regulation

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The first cloned chain (IFNAR1) of the human interferon-α (IFNα) receptor acts as a species-specific transducer for type I IFN action when transfected into heterologous mouse cells. Stably transfected mouse L929 cell lines expressing truncation mutants of the intracellular domain of the human IFNAR1 chain were tested for biological responses to human IFNα. Deletion of the intracellular domain resulted in a complete loss of sensitivity to the biological activity of human IFN but markedly increased IFNAR1 cell surface expression, demonstrating that the intracellular domain is required for biological function and contains a domain that negatively regulates its cell surface expression. Removal of the conserved membrane distal 16-amino-acid IRTAM (*I*nterferon *R*eceptor *T*yrosine *A*ctivation *M*otif) sequence: (1) increased sensitivity to IFNα's antiviral activity, (2) increased the rapid IFNα-dependent formation of STAT-containing DNA-binding complexes, (3) prolonged tyrosine phosphorylation kinetics of the JAK-STAT pathway, and (4) blocked the IFN-dependent down-regulation of the IFNAR1 chain. These results indicate that the IRTAM negatively regulates signaling events required for the induction of IFN's biological actions via regulating receptor down-regulation.

INTRODUCTION

IFNα is a cytokine with antiviral, antiproliferative, and immunomodulatory activities that binds to a multisubunit cell surface receptor shared with IFNβ (Branca *et al.,* 1982). The cloned components of the IFNα receptor, the IFNα receptor 1 (IFNAR1) and the IFNα receptor 2 (IFNAR2) subunits, are required for high-affinity IFNα binding and the IFNα-dependent activation of the JAK1 and TYK2 protein tyrosine kinases (Uze *et al.,* 1990; Novick *et al.,* 1994; Domanski *et al.,* 1995). IFN signaling to the cell nucleus involves the Jak kinase-mediated tyrosine phosphorylation of the latent cytoplasmic STAT (*s*ignal *t*ransducers and *a*ctivators of *t*ranscription) transcription factors. IFN-activated STAT1 and STAT2 translocate to the nucleus, where they recognize the conserved IFN stimulus response element (ISRE) within the promoter of IFN-stimulated genes (ISGs), which is both necessary and sufficient for ISG transcription (Schindler *et al.,* 1992; Fu, 1992). In addition, IFNα activates STAT3, which alone or in combination with STAT1 forms sis-inducible factors (SIFs) that bind to the c-sis-inducible element (SIE), originally described in the c-fos promoter (Wagner *et al.,* 1990).

The central importance of the IFNAR1 chain in the function of the type 1 IFN receptor has been demonstrated by the findings that the human IFNAR1 chain undergoes rapid ligand-dependent tyrosine phosphorylation and acts as a species-specific transducer for type I IFN action, and disruption of the IFNAR1 gene results in the loss of the antiviral response to type I IFNs (Cleary *et al.,* 1994; Muller *et al.,* 1994; Colamonici *et al.,* 1994a,b; Constantinescu *et al.,* 1994. The intracellular domains of type I IFN receptor chains contain conserved motifs that likely function in transmembrane signaling (Constantinescu *et al.,* 1994; Mullersman and Pfeffer, 1994). The intracellular domain of the human IFNAR1 (huIFNAR1) chain contains a membrane distal motif, KYSSQTSQDSGNYSNE, which is perfectly conserved in the mouse and bovine IFNAR1 chains (Uze *et al.,* 1990, 1992; Mouchel-Viehl *et al.,* 1992) and which we refer to as the *I*nterferon *R*eceptor *T*yrosine *A*ctivation *M*otif (IRTAM). Within the IRTAM are YSSQ and YSNE motifs which are potential docking sites for SH2-containing downstream effectors. These tyrosines are the only completely conserved tyrosine residues present in the intracellular domain of the IFNAR1 chain, and their phosphoyrlation is required for the IFN-dependent activation of STAT3 and PI3-kinase (Yang *et al.,* 1996; Pfeffer *et al.,* 1997b).

To determine the role that the IRTAM of the huIFNAR1 chain plays in type I IFN signaling, truncation mutants of

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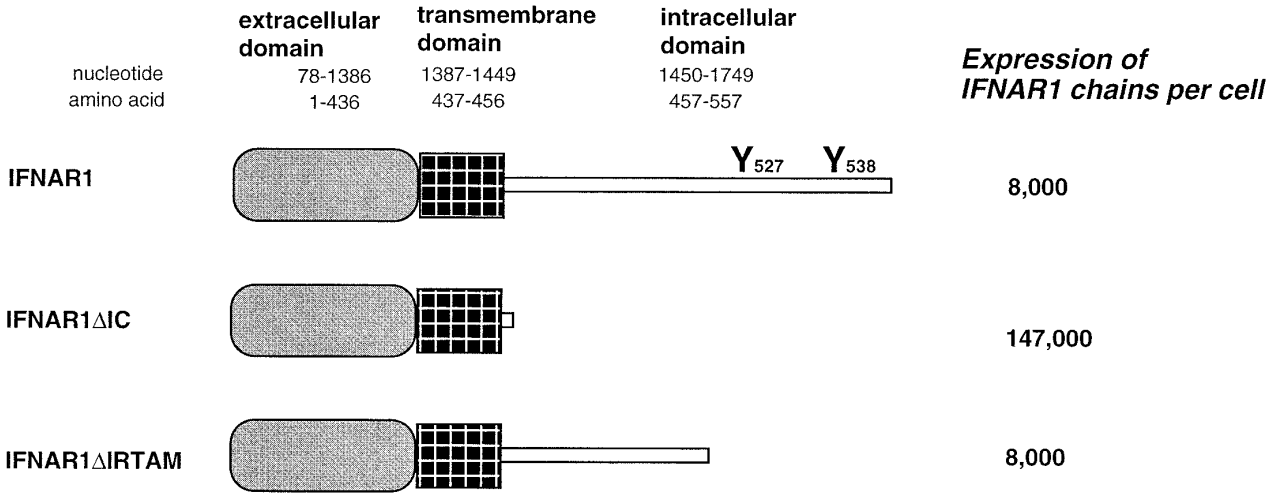

FIG. 1. Schematic diagram of truncation and point mutants of the huIFNAR1 constructs. The extracellular, transmembrane, and intracellular domains, as well as the two tyrosine residues in the IRTAM, are indicated. The number of huIFNAR1 chains in each transfectant was determined by binding assays performed with ^{125}I-labeled anti-huIFNAR1 The results of two separate assays on at least two independent transfectants performed in duplicate were averaged (SEM $<$ 15%).

the huIFNAR1 chain were generated. Mouse L929 cell transfectants expressing these mutant constructs were isolated and assayed for their biologic response to human type I IFN. The results demonstrate the critical role that the intracellular domain plays in IFN signaling and specifically identify the carboxyl-terminal region of the huIFNAR1 chain containing the IRTAM as an important regulatory element in IFNα signaling pathways.

RESULTS

Isolation of murine cell lines expressing wild-type or mutant constructs of the huIFNAR1 chain

In order to define the role of different domains of the human IFNAR1 chain in the biological response to human IFN, cDNAs encoding truncations in the intracellular domain of the huIFNAR1 were transfected into mouse L929 cells and stable cell lines were isolated. Figure 1 shows the cell surface levels of mutant or wild-type human IFNAR1 chains on transfectants that were selected for further study. Wild-type IFNAR1 and IFNAR1ΔIRTAM transfectants expressed similar levels of cell surface IFNAR1 chains (8000 sites/cell), while the IFNAR1ΔIC transfectant expressed extremely high levels of IFNAR1 chains ($\approx$150,000 sites/cell). Therefore, removal of the intracellular domain markedly increased IFNAR1 expression, while removal of the IRTAM had no effect on expression, indicating that a domain located membrane proximal to the IRTAM negatively regulates cell surface receptor expresion.

The antiviral effect of human IFN on cells expressing huIFNAR1 truncation mutant constructs

Since by definition IFNs inhibit viral replication, we determined the antiviral sensitivity to human IFN of murine cells expressing the various constructs of the huIFNAR1 chain. As shown in Fig. 2 and consistent with previous studies (Colamonici *et al.,* 1994b; Constantinescu *et al.,* 1995; Pfeffer *et al.,* 1997a), murine cells expressing the wild-type huIFNAR1 chain are sensitive to the antiviral effects of human IFNcon1 and IFNα8 on VSV replication. The antiviral effect in wild-type huIFNAR1-expressing transfectants exhibited a clear dose-dependence, with IFNcon1 showing a slightly higher antiviral activity than IFNα8 at all IFN concentrations examined. In contrast, cells transfected with the empty pLXSN vector of the IFNAR1ΔIC mutant were resistant to the antiviral action of human IFN, with little antiviral activity observed even with 10,000 IU/ml of human IFN. This result demonstrates the requirement for the intracellular domain of the huIFNAR1 chain to transduce the signal initiated at the receptor.

In contrast, cells transfected with the IFNAR1ΔIRTAM were more sensitive to the antiviral action of human IFNcon1 or IFNα8 when compared to cells expresing the wild-type huIFNAR1 chain, although the cell surface levels of the truncated receptor IFNAR1ΔIRTAM and wild-type huIFNAR1 were equivalent. For example, at an IFN concentration of 100 IU/ml there were 27.5 and $\approx$22-fold increases in the antiviral activity of IFNcon1 and IFNα8, respectively, in cells expressing the IFNAR1ΔIRTAM relative to the wild-type transfectants (Fig. 2). However, all transfected cell lines supported viral replication to similar extents (VSV titers between 1 and 3 $\times$ 10^8 PFU/ml), which argues against the differences in IFN sensitivities in the various transfectants being due to clonal variation.

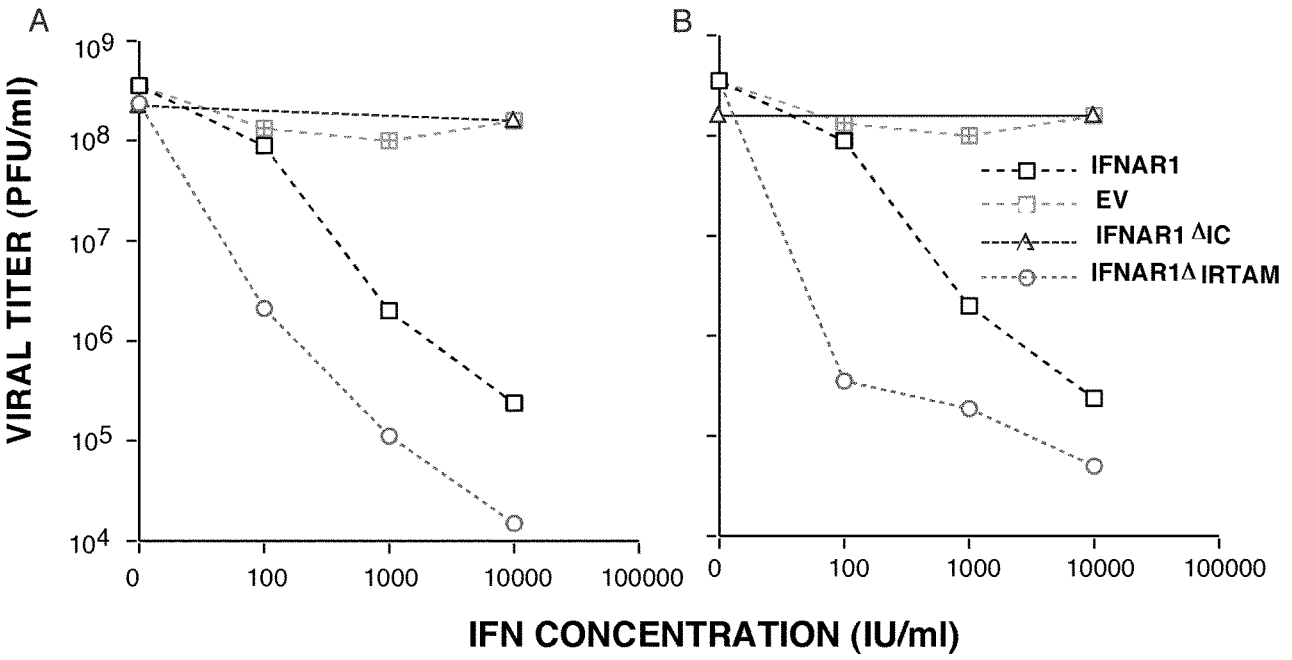

FIG. 2. The effects of human IFN on VSV replication in murine cells expressing wild-type or truncation mutants of the huIFNAR1 chain. Transfectants were treated for 18 h with IFNcon1 (A) or IFNα8 (B). IFN-treated cells were infected with VSV, and 24 h later the virus yield in the medium was assayed by plaque formation. The results of three separate experiments were averaged (SEM $<$ 20%) and expressed as the VSV titer produced. EV, empty vector transfectant.

IFN-dependent formation of STAT-containing ISGF3 and SIF complexes in cells expressing huIFNAR1 truncation mutants

Since removal of the IRTAM enhanced sensitivity to IFN's antiviral action, we examined the effect of the IRTAM's removal on the activation of the DNA-binding activity of STAT proteins. The IFN-induced activation of STAT1 and STAT2 in nuclear extracts of IFNcon1-treated transfectants was determined by ISGF3 formation in gel shift assays. As shown in Fig. 3A, ISGF3 activity was detectable in both wild-type huIFNAR1 and IFNAR-1ΔIRTAM transfectants following stimulation of cells with IFNcon1 for 30 min, was maximal at 2 h, and declined by 6 h. However, the ISGF3 activity was greater in the IFNAR1ΔIRTAM transfectants ($\approx$7.5- and 16-fold by quantitation of phosphorimages at 2 and 4 h, respectively). In contrast, IFN treatment did not induce ISGF3 formation in cells expressing the IFNAR1ΔIC or in cells transfected with the empty vector (Fig. 3B).

IFN treatment also activates SIF complexes (containing STAT1 and STAT3) which bind to the SIE present in various cytokine-regulated genes (Yang *et al.,* 1996). Since the pathways that result in the formation of ISGF3 and SIF complexes may be differentially regulated, we determined IFN-induced SIF formation in each of the mutant huIFNAR1 expressing cell lines. Figure 4A shows SIF formation at times varying from 0 to 24 h following IFNcon1 addition to wild-type huIFNAR1 transfectants, as assayed in gel shift assays with a labeled SIE oligonucleotide probe. A low basal level of SIF complexes was evident in untreated cells, which is in contrast to the failure to detect any basal levels of ISGF3 in the same cells. At 30 min following IFN stimulation SIF complexes

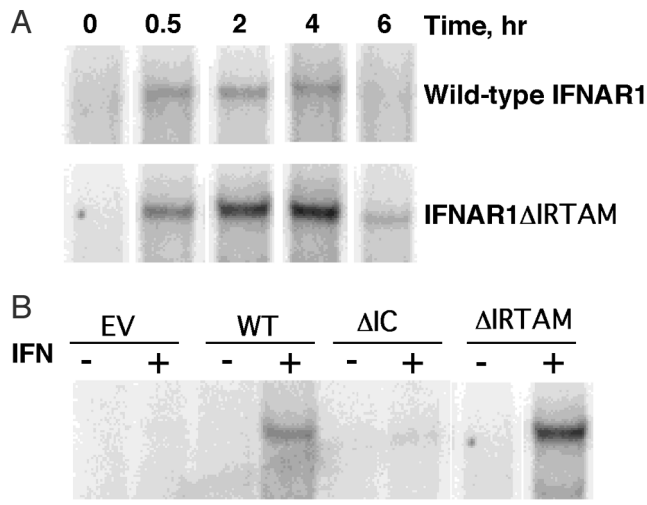

FIG. 3. IFN-induced ISGF3 formation in murine cells expressing truncation mutants of the huIFNAR1 chain. (A) Murine transfectants expressing the wild-type or the IFNAR1ΔIC construct (ΔIC) were treated with IFNcon1 (10,000 IU/ml) for times indicated prior to gel shift analysis with an ISRE probe. A representative result from three separate experiments is shown. (B) Murine L929 cells transfected with empty vector (EV), wild-type huIFNAR1 (WT), the IFNAR1ΔIC construct (ΔIC), or the IFNAR1ΔIRTAM (ΔIRTAM) were treated with IFNcon1 (10,000 IU/ml) for 30 min prior to gel shift analysis with an ISRE probe.

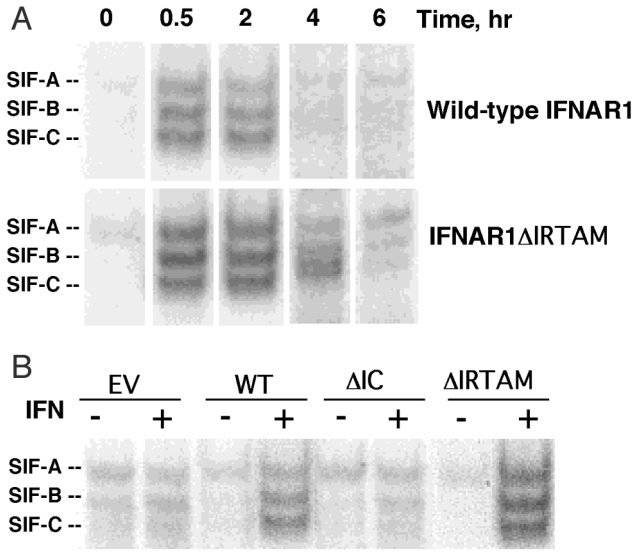

FIG. 4. IFN-induced SIF complex formation in murine cells expressing truncation mutants of the huIFNAR1 chain. (A) Murine transfectants expressing the wild-type or the IFNAR1ΔIC construct were treated with IFNcon1 (10,000 IU/ml) for times indicated prior to gel shift analysis with an SIE probe. A representative result from three separate experiments is shown. (B) Murine transfectants described in Fig. 3 were treated with IFNcon1 (10,000 IU/ml) for 2 h prior to gel shift analysis with an SIE probe.

were induced to near maximal levels, and they remained elevated at 2 h. During this time period the levels of SIF formation were greater in cells expressing the IRTAM deletion. The STAT compositions of the SIF complexes have been previously demonstrated by supershift assays (Yang *et al.,* 1996) to be STAT3 dimers (SIF-A), STAT1-STAT3 heterodimers (SIF-B), and STAT1 dimers (SIF-C). A higher IFN-dependent induction of all three SIF complexes was observed in the IFNAR1ΔIRTAM transfectants relative to the wild-type huIFNAR1-expressing cells (≈5- to 8-fold by quantitation of phosphorimages at 0.5 and 2 h, respectively). In contrast, the low basal level of SIF activity detected in the empty vector and IFNAR1ΔIC transfectants did not increase following IFN stimulation (Fig. 4B).

Prolonged activation of the JAK-STAT pathway in cells expressing IFNAR1ΔIRTAM

The induction of ISGF3 and SIF DNA-binding activity reflects the IFNα-dependent activation of the JAK1 and TYK2 protein tyrosine kinases and the Jak kinase-mediated tyrosine phosphorylation of STAT transcription factors. Since the levels of ISGF3 and SIF formation were greater in cells expressing the IRTAM deletion, we examined the IFN-dependent tyrosine phosphorylation of the TYK2 Jak kinase and the STAT2 transcription factor in wild-type and IFNAR1ΔIC transfectants. As shown in Fig. 5 TYK2 and STAT2 tyrosine phosphorylation was detectable in both wild-type huIFNAR1 and IFNAR1ΔIRTAM transfectants following stimulation of cells with IFNcon1 for 30 min. However, the levels of tyrosine phosphorylation of TYK2 and STAT2 were greater (~8- to 10-fold by quantitation of autoradiograms at 0.5 h) in the IFNAR1ΔIRTAM transfectants. Furthermore, the tyrosine phosphorylation of TYK2 and STAT2 persisted for longer times (greater than 6 h) after IFN treatment in the IFNAR1ΔIRTAM transfectants when compared to wild-type IFNAR1 transfectants (not detectable by 6 h for TYK2 and by 4 h for STAT2).

Impaired down-regulation of IFNAR1 in cells expressing IFNAR1ΔIRTAM

Since down-regulation of receptor subunits is a primary mechanism to terminate signal generation, we examined if the increased IFN sensitivity in IFNAR1ΔIRTAM transfectants reflected an impaired IFN-induced IFNAR1 down-regulation. IFN treatment (3 h) down-regulates ≈80% of the IFNAR1 chain in human cells and ≈50% of huIFNAR1 chains in mouse cells expressing human IFNAR1 chain (Constantinescu *et al.,* 1994). In the present study, the level of surface IFNAR1 in cells expressing the wild-type IFNAR1 and IFNAR1ΔIRTAM was determined following IFN treatment (3 h). Table 1 shows that IFN down-regulated >40% of huIFNAR1 chains in cells expressing wild-type IFNAR1 and in WA-17 mouse cells expressing three copies of the IFNAR1-encoding human

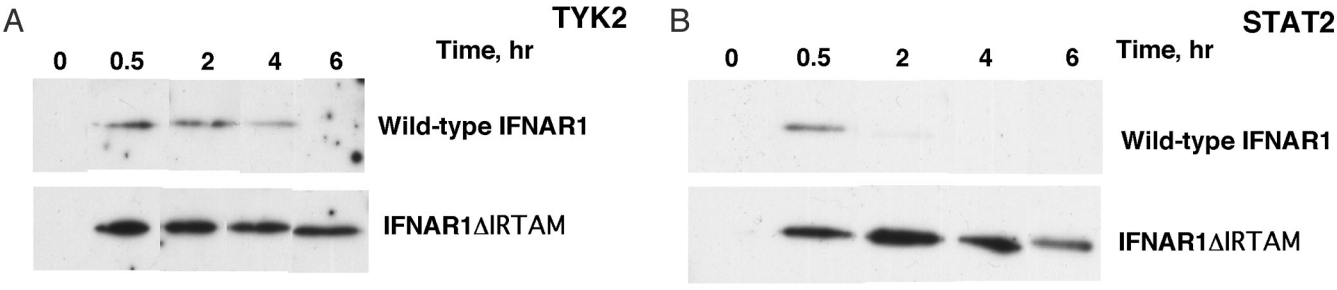

FIG. 5. IFN-induced tyrosine phosphorylation of TYK2 and STAT2 in murine cells expressing truncation mutants of the huIFNAR1 chain. Cell lysates were prepared from murine transfectants expressing the wild-type or the IFNAR1ΔIC construct treated with IFNcon1 (10,000 IU/ml) for times indicated. Lysates were immunoprecipitated with anti-TYK2 (A) or anti-STAT2 (B). The proteins were resolved by SDS–PAGE, blotted onto PVDF membranes, probed with anti-pTyr, and visualized by enhanced chemiluminescence (Amersham).

TABLE 1

IFNcon1-Induced Down-regulation of IFNAR1 Expression

Cell line	IFNAR1 sites	Percentage down-regulation induced by IFNcon1
WA-17 cell line	4200	46.3 ± 4.1
Wild-type IFNAR1 transfectant	8000	42.5 ± 2.6
IFNAR1ΔIRTAM transfectant	8000	0.5 ± 1.6

Note. Cells were treated for 3 h with IFNcon1 (10,000 IU/ml) and then the level of IFNAR1 expression was determined as described under Materials and Methods. Controls received no IFN. The results of four separate experiments were averaged.

chromosome 21. In contrast, no IFN-induced down-regulation of IFNAR1 chains was observed in the IFNAR1-ΔIRTAM transfectants.

DISCUSSION

Previous studies have established that the IFNAR1 chain plays a critical role in signal transduction by IFNα. Within minutes of IFNα exposure, the huIFNAR1 chain undergoes tyrosine phosphorylation (Constantinescu *et al.,* 1994), thereby providing phosphotyrosine docking sites for SH2 domain-containing cytoplasmic effectors. The intracellular domain of the huIFNAR1 chain has four potential tyrosine phosphorylation sites, which are apportioned equally into two intracellular subdomains: (1) a membrane proximal 50-amino-acid motif common to other signal transducing chains of the type II cytokine receptor family (Mullersman and Pfeffer, 1994), containing the docking site for TYK2 (Colamonici *et al.,* 1994a; Yan *et al.,* 1996b) and STAT2 (Yan *et al.,* 1996a); and the carboxy-terminal 16-amino-acid IRTAM with the only completely conserved tyrosine-based motifs in the huIFNAR1.

To define the role of the IRTAM in the biological function of IFNAR1, cell lines stably expressing various IFNAR1 truncation mutants were isolated. Consistent with previous studies, transfection of wild-type huIFNAR1 into mouse L929 cells made them sensitive to the antiviral and gene-inducing activities of human IFN, although L929 cells are intrinsically resistant to human IFN (Colamonici *et al.,* 1994b; Constantinescu *et al.,* 1994; Pfeffer *et al.,* 1997a). Most importantly, removal of the IRTAM increased responsiveness to human IFN, indicating that the IRTAM plays a negative regulatory role in IFN signaling. For example, the antiviral activity of human IFNcon1 and IFNα8 was greatly enhanced in mouse cells expressing the IFNAR1ΔIRTAM mutant of the IFNAR1 chain, when compared to cells expressing wild-type huIFNAR1. Furthermore, the levels of IFN-induced ISRE- and SIE-dependent gel shift activity were consistently greater in the IFNAR1ΔIRTAM transfectants than in the wild-type transfectants. These results are in agreement with the recent finding that removal of the IRTAM results in increased HLA class I induction and ISGF3 formation in murine transfectants expressing human IFNAR1 constructs (Gibbs *et al.,* 1996).

Furthermore, we provide evidence that the IRTAM can act as a negative regulatory domain, presumably by affecting receptor down-regulation. For example, we found that although IFN treatment (10,000 IU/ml for 3 h) down-regulated huIFNAR1 chains in wild-type transfectants, there was no detectable IFN-dependent down-regulation of the huIFNAR1 chain in the IRTAM truncation mutant. Blockage or delay of IFN-dependent down-regulation of the IFNAR1 chain would allow signals initiated by ligand binding to persist. Although we cannot exclude other mechanisms whereby the IRTAM can act as a negative regulatory domain (by steric hindrance, by acting as a pseudosubstrate, or by binding a tyrosine phosphatase), removal of the IRTAM enhanced IFN-dependent biological activities.

We also show that while the IFNAR1ΔIRTAM is biologically active, there is a complete loss of biologic activity in cells expressing a truncation of the huIFNAR1 intracellular domain. These results indicate that there are functionally important sequences in the intracellular domain of IFNAR1 between the transmembrane region and the membrane distal IRTAM. This is consistent with the fact that TYK2 and STAT2, both primary mediators of the type I IFN response, associate with the membrane proximal region of the huIFNAR1 chain (Colamonici *et al.,* 1994a; Yan *et al.,* 1996a,b). Since deletion of the IRTAM leads to a heightened sensitivity of murine transfectants to human IFN, important signaling molecules that primarily dock to the IRTAM of huIFNAR1 may also bind to alternative sites. For example, the YSSQ and YSNE motifs in the IRTAM of huIFNAR1 that bind STAT3 are also present in the murine IFNAR1 chain (Pfeffer *et al.,* 1997b). A YDDE motif (similar to the YSNE motif of the IRTAM) is present in the intracellular domain of the four cloned homologues of the full-length IFNAR2 chain (human, bovine, murine, and ovine). In addition, membrane proximal tyrosine residues (Y466 and Y481) of IFNAR1 may also serve as secondary docking sites for IFN-signaling molecules.

The antiviral response in the various huIFNAR1 transfectants directly correlated with the formation of STAT-containing DNA–protein complexes. While an increased antiviral activity of human IFN was observed in the IFNAR1ΔIRTAM transfectant in which an increased formation of IFN-dependent gel shift activities was also observed, the IFNAR1ΔIC transfectant did not respond to the antiviral action of human or to induction of ISRE- or SIE-dependent DNA–protein complexes. The role that IFN-induced proteins encoded by ISRE-controlled genes (Mx proteins, double-stranded RNA-dependent protein kinase, and 2,5-oligoadenylate synthetase) play in the antiviral effects of IFN are well documented. The role that SIF complexes play in IFN responses is unknown. How-

ever, since the SIE resembles the GAS element originally described in the promoters of IFNγ-stimulated genes, SIF complexes might mediate the transcription of a subset of IFNα-regulated genes which contain GAS elements, such as the IRF-1 transcription factor which is required for the antiviral action of IFN (Pine, 1992; Pine *et al.,* 1994).

In this report, we attempted to disentangle positive versus negative regulatory signals transduced through the IFNAR1 chain of the IFNα/β receptor. We separated changes in signaling due to differences in cell-surface expression (IFNAR1ΔIC) from effects due to more subtle alterations of IFNAR1 (IFNAR1ΔIRTAM) which do not modify the constitutive levels of expression. Our results suggest that the highly conserved 16-amino-acid IRTAM of huIFNAR1 chain transduces negative regulatory signals through the IFNα receptor by promoting IFN-induced IFNAR1 down-regulation. Removal of the IRTAM increased sensitivity to the antiviral activity of IFNα, increased the rapid IFNα-dependent tyrosine phosphorylation of TYK2 and STAT2, and increased the rapid IFNα-dependent formation of DNA–protein complexes that contain STAT1, STAT2, and STAT3.

MATERIALS AND METHODS

Interferons and monoclonal antibodies

The activity of recombinant human IFNcon1 (1×10^9 IU/mg) and IFNα8 (2×10^7 IU/mg) was assayed by protection against the cytopathic effect of vesicular stomatitis virus (VSV) on human fibroblasts and expressed in international reference units (IU/ml) using the NIH human IFNα standard for reference. The 4B1 MoAb directed against the extracellular domain of the huIFNAR1 chain has been previously described (Constantinescu *et al.,* 1994).

Cell culture and generation of stable transfectants

Mouse L929 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated bovine calf serum. Stable cell lines expressing the mutant constructs described were generated by liposome-mediated transfections. Plasmid DNA (9 μg) mixed with 54 μl of Lipofectamine (Life Technologies, Gaithersburg, MD) was added to 5×10^5 cells per 25-cm^2 flask. Transfected cultures were refed 24 h later and, after an additional 3 days, selected in medium containing 1.5 mg/ml G418 (Life Technologies). After 7 to 10 days, clones were isolated by limiting dilution.

Generation of truncation mutants of the huIFNAR1 chain

The huIFNAR1 truncation mutants were generated by PCR-mediated mutagenesis of p23 (American Type Culture Collection, Rockville, MD), which contains the IFNAR1 cDNA in pBluescript (SK$^-$) (Uze *et al.,* 1990). The intracellular truncation mutant IFNAR1ΔIC contained the complete extracellular and transmembrane domains and the first four residues of the intracellular domain to allow its anchorage in the plasma membrane. To generate IFNAR1ΔIC, PCR primers 5′-TTGAGTGAGTGAC-3′ and 5′-TCGAGTCACTCACTCAATGCA-3′ were annealed to generate a cassette encoding a stop codon in all three reading frames flanked by *Nsi*I- and *Xho*I-compatible ends. PCR reactions included 1 μM each primer, 100 μM each dNTP, 50 ng of p23, and 2.5 U of PFU polymerase in a 50-μl reaction volume. Fifteen amplification cycles were performed at 94°C for 1 min, 65°C for 1 min and 72°C for 2 min. The cassette was then cloned into the p23 IFNAR1 cDNA digested with *Nsi*I (nucleotide 1460 of the huIFNAR1 cDNA) and *Xho*I (present in the vector on the 3′ side of the cDNA). The huIFNAR1 mutant depleted of the IRTAM (IFNAR1ΔIRTAM) was generated by PCR using primers 5′-GCTGTATGTGAGAAAACAAAACCAGG-3′ and 5′-CTCGAGTCATTCTTCTACTGTAGCAATTGTGC-3′. The *Nsi*I/*Xho*I-digested PCR product was cloned into the *Nsi*I/*Xho*I-digested p23, replacing nucleotides 1348–2784 of the wild-type huIFNAR1 with the PCR product representing nucleotides 1348 through 1620. Constructs were sequenced using the dideoxy method (Sequenase; United States Biochemicals, Cleveland, OH) and cloned between the *Eco*RI and *Xho*I sites of the retroviral pLXSN vector (Miller *et al.,* 1993).

Analysis of huIFNAR1 expression by binding of I^{125}-labeled anti-IFNAR1 antibody

Cell lines expressing the transfected huIFNAR1 chains were screened by the binding of I^{125}-labeled 4B1 anti-huIFNAR1 MoAb as previously described (Constantinescu *et al.,* 1994). The number of huIFNAR1 chains expressed in each murine transfectant was determined by Scatchard analysis of the binding data. For down-regulation studies, cells were seeded at 2×10^5 cells per 2.1-cm^2 well (Constantinescu *et al.,* 1995). The next day, cells were treated for 3 h with 10,000 IU/ml IFNcon1, and the level of cell surface IFNAR1 chains was determined with I^{125}-labeled anti-huIFNAR1.

Antiviral assays

The antiviral activity of human IFNs was measured by the inhibition of VSV replication in mouse cells (Improta *et al.,* 1992). Transfectants (5×10^6 cells per 25-cm^2 flask) were treated with IFN for 1 day and then infected at 0.1 plaque-forming unit (PFU)/cell of VSV for 2 h. At 24 h postinfection, virus production was assayed by plaque formation on Vero indicator cells (Pfeffer *et al.,* 1990).

Gel shift assays

For gel shift analysis, equivalent amounts of the nuclear extracts prepared as previously described (Yang *et al.,* 1996) were incubated with a ^{32}P-labeled promoter probe for either the high-affinity SIE from the c-fos gene (5′-GTCGA-CATTTCCCGTAAATC-3′) or the ISRE from ISG15 (5′-GATCGGGAAAGGGAAACCGAAACTGAAGCC-3′) at 20°C for 20 min, and the free probe was separated from protein–DNA complexes on 5% polyacrylamide gels.

Immunoprecipitations and immunoblot analysis.

For immunoprecipitation studies, cultures were washed with ice-cold PBS and lysed for 20 min in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 % NP-40; 15% glycerol) containing 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 1.75 μg/ml benzamidine (Fu, 1992). Samples were centrifuged (12,000 *g,* 15 min) at 4°C and supernates were immunoprecipitated with anti-TYK2 or -STAT2 Ab (Santa Cruz Laboratories) overnight at 4°C. Immune complexes were collected using Protein A–Sepharose beads (Pharmacia) and eluted in sample buffer. Samples were run on SDS–7.5% PAGE, transferred to PVDF membranes (Millipore), and probed with anti-phosphotyrosine (anti-pTyr, Oncogene Sciences Ab-2) (dilution 1:1000), followed by anti-mouse IgG coupled with horseradish peroxidase (Amersham). Blots were developed using enhanced chemiluminescence (ECL, Amersham).

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