Induction of β-R1/I-TAC by Interferon-β Requires Catalytically Active TYK2*

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The β-R1/I-TAC (interferon-inducible T-cell α-chemoattractant) gene encodes an α-chemokine that is a potent chemoattractant for activated T-cells. We previously reported that β-R1 was selectively induced by interferon (IFN)-β compared with IFN-α and that the canonical type I IFN transcription factor interferon-stimulated gene factor 3 (ISGF3) was necessary but not sufficient for β-R1 induction by IFN-β. These findings suggested that β-R1 induction by IFN-β required an accessory component. To begin characterizing this signaling pathway, we examined the function of TYK2 protein in the IFN-β-mediated induction of β-R1. This study was motivated by the observation that β-R1 could not be induced in TYK2-deficient U1 cells by IFN-β (Rani, M. R. S., Foster, G. R., Leung, S., Leaman, D., Stark, G. R., and Ransohoff, R. M. (1996) J. Biol. Chem. 271, 22878–22884), an unexpected result because IFN-β evokes substantial expression of IFN-stimulated genes (ISGs) in U1 cells through a TYK2-independent pathway. We now report β-R1 expression patterns in U1 cells complemented with wild-type or mutant TYK2 proteins. Complementation with wild-type TYK2 rescued IFN-β-inducible expression of β-R1. Cells expressing kinase-deficient deletion or point mutants of TYK2 were refractory to induction of β-R1 by IFN-β despite robust expression of other ISGs. Transient transfection analysis of a β-R1 promoter-reporter confirmed that transcriptional activation of β-R1 by IFN-β required competent TYK2 kinase. These studies indicate that the catalytic function of TYK2 is required for IFN-β-mediated induction of β-R1. Catalytic TYK2 is the first identified component in an accessory signaling pathway that supplements ISGF3/interferon-stimulated response element signaling for gene induction by type I IFNs.

Interferons (IFNs)1 elicit multiple biological responses in-
IFN-α2 (12–15). This result suggested that IFN-β engagement generated a more stable signaling complex than IFN-α2. Recently, it has been shown that IFN-α2 and IFN-β require distinct intracytoplasmic regions of the IFNAR-2 chain of the receptor to elicit an antiviral response (15).

We recently reported studies of the regulation of a gene designated β-R1, which was selectively induced by IFN-β compared with IFN-α in astrocytoma and fibrosarcoma cells (16). Sequence analysis of cDNAs indicated that β-R1, which was initially cloned by differential display, was predicted to encode a chemokine-like peptide with an N-terminal CXC motif. Independently, Neote and colleagues (17) isolated a chemokine designated I-TAC from cytokine-treated fetal astrocytes. Initial analysis of multiple independent cDNA isolates has established identity between the two chemokines. β-R1/I-TAC possesses potent chemotactic activity toward activated T-lymphocytes (17).

The signaling pathway whereby IFN-β induces the expression of β-R1 appears to be novel. Our studies, conducted in fibrosarcoma cell lines that were deleted for individual constituents of the IFN signaling pathway, established the following: 1) β-R1 was selectively induced by IFN-β in comparison to IFN-α2, IFN-αCON, mixtures of IFN-α subtypes, or IFN-αβ; 2) cellular components needed to generate transcription factor ISGF3 were essential but not sufficient for induction of β-R1 by IFN-β; 3) the ISRE-binding protein p48 was essential for β-R1 expression in response either to IFN-β or IFN-γ, implying that transcription was regulated by an ISRE-like element (16).

Unexpectedly, U1 cells that lacked TYK2 expression failed to express β-R1 in response to IFN-β; this result was confirmed in three unrelated lines of U1 cells obtained from two independent mutagenesis experiments (16). This finding was surprising as U1 cells are responsive to IFN-β for ISGs, through a TYK2-independent pathway (18, 19). Furthermore, when U1 cells were immunoselected for high efficiency response to IFN-β using major histocompatibility complex I induction to monitor the IFN response, β-R1 induction was not rescued (16). These results suggested that TYK2 protein mediated an essential structural or catalytic role in the induction of β-R1 by IFN-β.

TYK2 is a 135-kDa cytosolic protein characterized by the presence of a C-terminal protein tyrosine kinase (TK) domain and an adjacent kinase-like (KL) domain. Five further domains of substantial amino acid similarity with other JAKs extend to the N terminus of the protein and are designated JAK homology α-β domains (Fig. 2A) (20, 21).

The functions of receptor-associated tyrosine kinases TYK2 and JAK1 in type I IFN signaling pathway have been well established in part through the study of IFN-α unresponsive human fibrosarcoma mutant cell lines U1 and U4 lacking TYK2 and JAK1, respectively (18, 22, 23). Both kinases interact in ligand-independent fashion with type I IFN receptor components, TYK2 associated with IFNAR1 (24–26) and JAK1 with IFNAR2c (27, 28). JAK1 catalytic activity is absolutely essential for responses to type I IFNs (22). In contrast, IFN-β signaling for induction of ISGs can proceed at reduced efficiency in the absence of TYK2, and kinase-deficient TYK2 protein augments responses to type I IFNs in U1 cells (18, 29).

Prior reports delineated various functions for TYK2 in IFN signaling. First, TYK2 protein is required to sustain expression of IFNAR1; the complete failure of U1 cells to respond to IFN-α species correlated with the inability to bind ligand (18). The contribution of TYK2 to the ligand binding activity of type I IFN receptor does not require catalytic activity. Indeed, ligand-binding function for IFN-α is restored by expression of kinase-deficient TYK2 (20). Second, TYK2 is required for generating STAT-docking sites on IFNAR1. Docking occurs through mutual phosphotyrosine/src-homology-2 (SH2) interactions between Tyr-466 on the activated IFNAR1 cytoplasmic tail and STAT-2 which is preassociated with IFNAR2c (30). This SH2-phosphotyrosine interaction determines signaling specificity through the type I IFN receptor complex (31). The role of TYK2 in this process was demonstrated by showing that TYK2 phosphotyrosylates IFNAR1 in vitro and that IFNAR1-Tyr-466-containing phosphopeptides block signaling (25, 26).

In this report, we describe further investigation of the role of TYK2 in the induction of β-R1, using U1 cells complemented with TYK2 deletion and substitution mutants or with wild-type TYK2. Kinase-deficient TYK2 mutant proteins restored IFN-α binding and weak response to IFN-α; responses to IFN-β were augmented (20). However, none of the cells complemented with mutant binding TYK2 proteins expressed β-R1 in response to IFN-β, indicating that TYK2 catalytic activity was required for the induction of β-R1.

### MATERIALS AND METHODS

**Cells and Interferons**

Human fibrosarcoma 2fTGH cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (18). U1.wt, U1.KR930, U1.YFF1054–55, U1.ΔTK, and U1.ΔKL were maintained in Dulbecco’s modified Eagle’s medium with 10% calf serum in the presence of 250 μg/ml hygromycin and 450 μg/ml G418 (20). Purified recombinant IFN-α2 (1 x 10⁶ units/ml) was obtained from Wellcome, and recombinant IFN-β1b (2 x 10⁶ units/mg protein) was from Berlex Biosciences (Richmond, CA). IFNs were used at a final concentration of 1000 units/ml unless stated otherwise.

**Cloning the Promoter of β-R1**

A human genomic library (CLONTECH) was screened using the partial β-R1 cDNA obtained by RNA fingerprinting (16). Seven clones were obtained. Southern blot analysis of phage DNA digested with restriction enzymes EcoRI and SalI revealed a 3-kilobase pair fragment that hybridized with a 100-bp probe to the extreme N terminus of the cDNA. The DNA from one clone was cleaved with EcoRI, and fragments were randomly cloned into pBS vector. A clone containing a 3-kilobase pair insert was determined by sequence analysis to contain 300 bp of sequence content upstream of the putative transcription start site of β-R1, by alignment with cDNA products.
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Transient Transfection Assay

Plasmids—A 303-bp upstream putative β-R1 promoter element including the transcription start site was amplified by polymerase chain reaction using Fβ-R1 containing the SacI restriction site (5’AGGCGGAGCTTCCCGTCCCCG) and Rβ-R1 containing the BglII restriction site (5’TGGAAAGATCTAGTAAAGT) incorporated in the primers. The PCR-amplified product was excised with BglII and SacI and was subcloned into the promoterless plgG3-basic vector (Promega Corp., Madison, WI). One clone (pβ-R1-300-luc) was sequenced to verify the nucleotide sequence and has the 5’-flanking region of the β-R1 gene from -305 to +1 (transcriptional start site), and DNA from this clone was used for transfection experiments.

A 102-bp promoter-reporter construct containing the ISRE from the p56 ISG was provided by Dr. Ganes Sen (Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH). A simian virus 40 promoter-β-galactosidase reporter plasmid (pCH110, Amersham Pharmacia Biotech) was co-transfected with experimental plasmids as an internal control to normalize for transfection efficiency.

Transfection—293 cells, U1 wt-5, and U1LKB1930 cells were grown to 70–80% confluency in 100-mm plates, co-transfected with 10 µg of test plasmid DNA and 1.5 µg of pCH110 β-galactosidase plasmid DNA using Polybrene (10 µg/ml) for 6 h at 37°C. After incubation, the cells were subjected to Me2SO shock for 90 s (30% Me2SO in Dulbecco’s modified Eagle’s medium), washed, and allowed to recover overnight from Me2SO shock. The following morning, cells were pooled and equally redistributed in several plates and reserved as controls or treated with IFN-β (1000 units/ml) for 16 h. Lysates were prepared, and β-luciferase activity was assayed using a luciferase assay kit (Promega Corp., Madison, WI), and measurements made using a Luminometer (Dynatech Laboratories, Chantilly, VA). β-Galactosidase activity was assayed using Galacto-Light Plus assay (Tropix Inc., Bedford, MA). Equal amounts of protein were assayed for enzyme expression, and luciferase activity was normalized to β-galactosidase activity.

Cell Extracts and EMSA

Cells were treated with IFNs or reserved as controls, harvested, and lysed for 15 min on ice in hypotonic buffer containing 20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na3VO4, 1 mM Na3P2O7, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonfluoride, leupeptin (1 µg/ml), aprotonin (1 µg/ml), and pepstatin (1 µg/ml). The extract was centrifuged at high speed for 1 min. The pellet was discarded and 0.1 M NaCl added to the supernatant and microcentrifuged for 1 min and adjusted to 10% glycerol. Protein concentration was measured by the method of Bradford by using protein dye reagent (Bio-Rad). Extracts were stored at -70°C until use.

For binding reactions, extracts (10 µg of protein) were incubated with a [32P]-labeled oligonucleotide probe (50 ng, 20 000–40 000 cpm) in buffer containing 6 mM HEPES, 1 mM dithiothreitol, 6% glycerol, and 5 µg of poly(dI-dC) for 20 min at room temperature. An oligonucleotide probe corresponding to ISRE sequence of the 6-16 gene, 5’CTCTTCTGG-GAAAATGAAACTCA3’ was used. The reaction products were resolved on 8% non-denaturing polyacrylamide gels which were dried and analyzed by autoradiography.

RNase Protection Assay

Total RNA was prepared from IFN-treated cells using the Trizol method (32), and protection experiments were performed as described (16). The probes used were β-R1 (protects 500 bases), 6-16 (protects 190 bases), and 9-27 (protects 160 bases) (16, 33). γ-Actin probe protects 130 bases, and IRF-1 probe protects 175 bases; these are as described by Muller et al. (34). The hybridization signal was quantitated with a PhosphorImager (Molecular Dynamics). To compare the response to IFN-β with IFN-α, β-R1 induction was normalized to the induction of ISG, as shown.

β-R1 induction ratio (IFN-β/IFN-α): 6-16(α)/γ-actin × β-R1(β)/β-R1(α)

TABLE II

| IFN    | IFN-β/IFN-α induction ratio for β-R1 |
|--------|--------------------------------------|
| 800    | 6.8                                  |
| 1600   | 9.6                                  |
| 3200   | 9.9                                  |
| 6400   | 7.6                                  |

The densitometric ratio of β-R1, normalized to 6-16/γ-actin at different concentrations of IFN (Fig. 1), was determined after the protected fragments from the RPA assay were quantitated on a PhosphorImager (Molecular Dynamics). To compare the response to IFN-β with IFN-α, β-R1 induction was normalized to the induction of ISG, as shown.

The role of TYK2 in induction of β-R1 was examined by studying U1 cell lines complemented with wild-type or mutant TYK2. The previously reported characteristics of these cell lines, all derived from HT1080 fibrosarcoma cells, are summarized in Table I. In CRT astrocytoma cells (from which β-R1 was originally cloned), β-R1 was induced to an equivalent extent by 10 units/ml IFN-β or by 2500 units/ml IFN-α2 (16). To initiate our studies in cells of the HT1080 background, we examined differential regulation of β-R1 by IFN-β compared with IFN-α in these cells. Dose-response experiments in the HT1080 cells indicated that selective induction of β-R1 by IFN-β was maintained over a range of IFN concentrations studied (Fig. 1 and Table II). In order to compare β-R1 induction by IFN-α2 with IFN-β, we normalized the IFN response to induction of the ISG 6-16 (Table II, see legend).

By this analysis, IFN-β was an average of 8–9 fold more efficient for induction of β-R1 than IFN-α at concentrations of 800–6400 units/ml. The differential response to IFN-β was not observed for 6-16, a well characterized type I IFN-induced gene.

The differential regulation of β-R1 mRNA by IFN-β as compared with IFN-α could be determined at the level of transcriptional or post-transcriptional events. In order to begin addressing this issue, we examined the stability of β-R1 mRNA induced by IFN-α2 or IFN-β. A high dose of IFN-α2 (2500 units/ml) was used to induce β-R1 and was compared with 2500 units/ml IFN-β. After cells were treated with IFNs for 6 h, actinomycin D (5 µg/ml) was added to arrest transcription, and β-R1 mRNA levels were analyzed by normalized nucleic acid protection assays at hourly intervals up to 8 h (the time point of maximal accumulation of the message). β-R1 mRNA was equally stable up to 8 h after arrest of transcription in cells treated with either IFN-α2 or IFN-β, arguing that differential stability of β-R1 message did not account for the accumulation of β-R1 mRNA in cells treated with IFN-β compared with...
**Fig. 2.** A, schematic of the structure of TYK2 mutant proteins. Names of the proteins are on the left. *wt*, wild-type; *KL*, kinase-like domain; *TK*, tyrosine kinase domain. B, induction of β-R1 by IFN-β in cells expressing wild-type or TYK2-deleted/substituted forms of protein. The figure shows an autoradiogram derived from one representative experiment (out of five) using RNase protection assay of total RNA (20 μg) from untreated U1.**

**Fig. 3.** Induction of β-R1, ISGs 6-16, 9-27 in U1.wt-5, U1.KR930 and U1.YYFF1054–55 cells by 10,000 units/ml recombinant IFN-β for 16 h. RNase protection assay of total RNA (20 μg) was performed, and the densitometric ratio of β-R1, 6-16, and 9-27 to γ-actin was determined after protected fragments were quantitated by NIH image analysis. Results from one of two experiments are shown.
IFN-α2 (results not shown).

Induction of β-R1 in Cells Expressing TYK2 Deletion and Substitution Mutants—To determine the role of catalytic TYK2 in the induction of β-R1, we examined regulation of this and other ISGs in U1 cell lines expressing wild-type TYK2 or mutants that are schematically depicted in Fig. 2A. Deletion mutant ΔTK lacks the tyrosine kinase domain, whereas ΔKL lacks the kinase-like domain but retains an intact tyrosine kinase domain. Both deletion mutants lack kinase function in vitro (20). The mutant KR930 was constructed by substituting lysine for arginine in the ATP-binding site that results in the generation of a kinase-inactive TYK2 protein that retains weak ligand-dependent phosphorylation on tyrosine (Table I) (29). In substitution mutant YYFF1054–55, two conserved tyrosines are mutated to phenylalanine in the putative activation loop. Phosphorylation of these tyrosines is required for ligand-dependent activation of TYK2. Therefore, the YYFF1054–55 substitution mutant YYFF1054–55, two conserved tyrosines in the induction of ISGs (Fig. 3). Strikingly, β-R1 was not induced in U1.KR930 and U1.YYFF1054–55 cells was detected on prolonged exposure of autoradiograms (Fig. 3).

To address the possibility that inability to induce β-R1 by IFN-β in cells expressing mutant TYK2 proteins could result from decreased signaling efficiency, cells were exposed to higher concentrations of IFN-β. At the highest concentration of IFN-β tested (10,000 units/ml), no induction of β-R1 by IFN-β in U1.KR930 and U1.YYFF1054–55 cells was detected on prolonged exposure of autoradiograms (Fig. 3).

Induction of 6-16 by either IFN-α2 or IFN-β was equally robust in cells expressing either kinase-deficient or wild-type TYK2. ISG 9-27 also accumulated in U1.KR930 and U1.YYFF1054–55 in response to either IFN-α2 or IFN-β (Fig. 3). These results supported a specific role for inducible TYK2 catalytic activity in regulating expression of β-R1 in response to IFN-β.

Overexpression of TYK2 Selectively Augments Induction of β-R1 by IFN-β—Cells expressing endogenous or increased levels of TYK2 (2TGH and U1.wt) were compared for inducible expression of β-R1 or other ISGs (Fig. 4). β-R1 mRNA accumulated approximately 9-fold more in U1.wt-5 cells than in 2TGH, in response to IFN-β (Fig. 4). The induction of other ISGs (6-16 and 9-27) varied at most by 2-fold in U1.wt-5 cells.

FIG. 5. Generation of ISGF-3 is not defective in U1.KR930 cells. EMSA using whole cell extracts from 2TGH, U1.wt-5, and U1.KR930 cells treated with or without (C) 2500 units/ml recombinant IFN-α or IFN-β for 15 min and incubated with a oligodeoxynucleotide probe containing the ISRE of 6-16 gene. DNA-protein complexes were separated by native polyacrylamide gel electrophoresis (%J) and analyzed by autoradiography.

Pressing both wild-type and mutant TYK2 (Fig. 2B). Strikingly, β-R1 was not induced by IFN-β treatment in either deletion mutants (U1.ΔTK and U1.ΔKL) or substitution mutants (U1.YYFF1054–55 and U1.KR930) of TYK2 in response to IFN-β (Fig. 2B).

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Fig. 7. β-R1 transcription is defective in U1.KR930 cells. Transient transfection analysis of pβ-R1-300-luc construct and p561-luc construct in U1.wt-5 cells and U1.KR930 cells exposed to 1000 units/ml recombinant IFN-β for 16 h. The histograms show means (± S.D.) for five experiments with pβ-R1-luc 300 and three experiments with p561-luc. Fold induction by IFN-β of pβ-R1 300-luc in U1.wt-5 and U1.KR930 cells differed significantly (p < 0.001, paired t test), and expression of pβ-R1 300-luc in U1.KR930 cells did not differ significantly from untreated controls.

Compared with 2TGH, IFN-induced Generation of ISGF3 Is Not Defective in U1.KR930 Cells—Failure of β-R1 induction in TYK2-deficient cells could result from impaired generation of transcription factor ISGF3, whose components we previously showed to be essential for transcription of β-R1 (16). Expression of ISGs strongly suggested the formation of ISGF3 in U1-derived cells complemented with kinase-deficient TYK2; however, the presence of ISGF3 had not been formally demonstrated. To address this issue, electrophoresis mobility shift assay (EMSA) was used to monitor generation of ISGF3 after IFN treatment. The composition of this complex has been previously confirmed with supershift analysis, using anti-STAT antibodies (35). In 2TGH cells, the major type I IFN subtypes (IFN-α2 or IFN-β) generated approximately equal abundance of ISGF3 complex, indicating that the presence of ISGF3 was not sufficient for induction of β-R1 (Fig. 5, lanes 2 and 3). After IFN-β treatment, 2TGH cells and U1.KR930 cells contained equivalent amounts of ISGF3, yet only 2TGH cells expressed β-R1, further supporting this interpretation (Fig. 5, lanes 3 and 7). In U1 cells, ISGF3 was generated weakly in response to IFN-β but not IFN-α2, consistent with induction of ISGs by IFN-β in these cells (results not shown).

IFN-β Induces β-R1 Transcription in U1.wt-5 But Not U1.KR930 Cells—To investigate the mechanism by which IFN-β induces β-R1 expression, a 303-bp fragment of the human β-R1 gene (Fig. 6), upstream of the transcription start site, was subcloned into the promoterless pGL3 plasmid, producing the promoter-reporter construct pβ-R1-300-luc, in which expression of the luciferase reporter gene was controlled by cloned β-R1 sequence. The sequence content of this putative promoter element was examined for potential regulatory elements; two GAS sites and one binding site each for AP-1, NF-AT, and NFκB, and one ISRE-like element were identified (Fig. 6).

Normalized transient transfection analysis was used to determine the function of this element in U1.wt-5 and U1.KR930 cells. pβ-R1-300-luc was significantly induced by IFN-β in U1.wt-5 but completely inert in U1.KR930 cells, demonstrating that this fragment contained a functional β-R1 promoter that was dependent on catalytic TYK2 for its induction (Fig. 7). A promoter-reporter from the 561 ISG was induced by IFN-β approximately twice as efficiently in U1.wt-5 as in U1.KR930 cells and retained significant activity in cells expressing kinase-deficient TYK2 (Fig. 7). These results reflected the relative IFN-β-mediated induction of endogenous β-R1 and ISGs in U1.wt-5 and U1.KR930 (compare Figs. 3 and 4 with Fig. 7) and indicated that the differential accumulation of the respective mRNAs was regulated at the transcriptional level.

DISCUSSION

Our current results suggest that catalytically active TYK2 is required for accessory signaling that results in transcription of β-R1 in IFN-β-treated cells. Addressing this point most directly, β-R1 was not inducible in U1 cell lines complemented with various deletion or substitution mutants of TYK2, each of which were catalytically inactive.

Furthermore, IFN-β-mediated transcription of β-R1 requires inducible TYK2 kinase activity, as shown by failure of induction in cell lines expressing U1.KR930 and U1.YYFF1054–55 mutants. Both proteins lack ligand-inducible kinase activity, whereas the U1.YYFF1054–55 mutant retains basal kinase function, indicating that this residual enzymatic action is insufficient to mediate β-R1 induction. Recent reports also suggest that SH2-phosphotyrosine interactions are important for association between STAT substrates and JAKs in type I IFN signaling (36). Our results suggest that the docking function of the TYK2 protein (absent inducible kinase activity) may not be sufficient for β-R1 expression, since IFN-β could not induce this gene in U1.KR930, expressing a TYK2 mutant that undergoes phosphorylation in trans on tyrosine residues (29).

The failure of IFN-β to induce β-R1 in cells expressing kinase-deficient TYK2 contrasted with IFN-β-mediated expression of other ISGs, activation of ISGF3, and establishment of the antiviral state (not shown) in U1 cells complemented with catalytically inactive TYK2. These results extend prior demonstrations that IFN-β can utilize a TYK2-independent pathway (19) for induction of ISGs. Despite the availability of TYK2-independent IFN-β-mediated signaling, generation of docking sites on IFNAR1 (a TYK2-dependent function) clearly enhances the efficiency of IFN signaling. However, we did not detect β-R1 induction by IFN-β in cells expressing kinase-deficient forms of TYK2 at high concentrations of ligand that strongly induced expression of other ISGs and efficient antiviral responses in U1.KR930 cells (not shown). This observation suggested a specific role for TYK2 in the signaling pathway for β-R1 transcription. As compared with 2TGH cells, IFN-β treatment of U1.wt-5 cells that overexpressed TYK2 resulted in
selective increase in accumulation of β-R1 mRNA, further supporting a specific role for TYK2 in β-R1 induction.

A 303-bp β-R1 genomic fragment was isolated for these studies, and its function was examined in transient transfection assays. The pβ-R1-300-luc plasmid directed luciferase expression in response to IFN-β in U1.wt-5 but not U1.KR930 cells, precisely reiterating the pattern of expression observed for the endogenous gene and indicating that TYK2-dependent regulation of β-R1 by IFN-β occurs at the transcriptional level.

In summary, data described in this report demonstrate that the pathway utilized by IFN-β for inducing the β-R1 gene requires catalytically active TYK2. Elucidation of the components and mechanisms of this accessory pathway will provide insights into biological functions of IFNs for which JAK-STAT signaling is essential but not sufficient.

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