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Biochemistry

Tyrosine phosphorylation is required for activation of an α interferon-stimulated transcription factor

(signal transduction/gene expression)

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ABSTRACT **The signal transduction pathway of α interferon utilizes tyrosine phosphorylation to transmit a signal generated at the cell surface to the transcriptional machinery in the nucleus. Activation of the interferon pathway initiates with the binding of α interferon to its cell surface receptor. The ligand–receptor complex signals the activation of a latent cytoplasmic transcription factor. The active form of the interferon-stimulated gene factor (ISGF3) is phosphorylated on tyrosine residues. ISGF3 subsequently translocates to the nucleus and binds to a DNA sequence, the interferon-stimulated response element, found within the promoter of inducible genes. ISGF3 is a multicomponent factor consisting of four proteins of 113 kDa, 91 kDa, 84 kDa, and 48 kDa. Three proteins consistent with sizes of 113 kDa, 91 kDa, and 84 kDa copurify with ISGF3 and are phosphorylated on tyrosine residues after stimulation by α interferon. Tyrosine phosphorylation is essential for activation of ISGF3. Genistein, a tyrosine kinase inhibitor, blocks the appearance of ISGF3 and blocks the transcriptional stimulation of interferon-induced genes. This study shows that tyrosine phosphorylation provides a link between the interferon–receptor complex at the plasma membrane and specific activation of gene expression in the nucleus.**

Elucidation of signal transduction pathways that initiate at the plasma membrane and culminate in transcriptional responses in the nucleus has been the focus of a wide range of investigations. The α/β interferons (IFN-α/β) are a family of cytokines that alter the physiological state of the cell and lead to a resistance to viral infection and an inhibition of cellular proliferation (reviewed in refs. 1–3). IFN-α/β elicits these responses by binding to a specific cell surface receptor and inducing the transient expression of a subset of genes (reviewed in refs. 4 and 5). These IFN-stimulated genes (ISGs) possess within their promoter a specific DNA response element, the IFN-stimulated response element (ISRE) (6–11). The ISRE is an inducible enhancer that is both necessary and sufficient for the transcriptional response to IFN-α/β (12, 13). The ISRE is recognized by a unique DNA-binding transcription factor that rapidly appears after IFN binds to its receptor. This IFN-stimulated gene factor, ISGF3, preexists in a latent form in the cytoplasm of the cell and becomes activated after IFN treatment (14, 15). Activation of ISGF3 results in its ability to bind to the ISRE and stimulate transcription.

ISGF3 is a multimeric transcription factor composed of four proteins (16, 17). One component is a 48-kDa protein that preexists in low levels in the cell but can be induced to higher levels with IFN-γ. It is referred to as the ISGF3γ subunit. ISGF3γ alone possesses a weak affinity for the ISRE. Three other proteins of 113 kDa, 91 kDa, and 84 kDa form a complex referred to as the ISGF3α subunit. Treatment of cells with IFN-α/β leads to the activation of ISGF3α and its association with ISGF3γ to form the functional ISGF3 (15, 18).

A gene encoding the IFN-α/β receptor has been isolated and characterized (19). However, its characterization has not provided information elucidating a signal transduction mechanism. It appears likely that the IFN–receptor complex associates with a distinct transducing component or subunit (20, 21). Previous studies have shown that staurosporine, a nonspecific but potent protein kinase C and tyrosine kinase inhibitor, blocks activation of the IFN-α/β signal transduction pathway (22–24). In this report we provide evidence for the involvement of a tyrosine kinase in gene activation by IFN-α/β. The latent transcription factor, ISGF3, is a substrate for an IFN-stimulated tyrosine kinase and serves as a signal transducer to the nucleus. The tyrosine-phosphorylated ISGF3 binds to the ISRE and leads to the activation of specific gene transcription.

MATERIALS AND METHODS

Cell Culture. Human HeLa S3 cells were maintained as monolayers in Dulbecco's modified Eagle's medium containing 8% (vol/vol) fetal bovine serum or in suspension in minimal essential medium containing 2% fetal bovine and 3% calf serum. Cultures were treated with 500–1000 units of human IFN-α2a per ml or 50 units of human IFN-γ per ml, both provided by Hoffmann–La Roche. The tyrosine kinase inhibitor genistein (GIBCO/BRL) was used in a 12-hr treatment at 100 μg/ml.

Transcription Rates. Nuclear run-on analyses were performed as described (7, 22). Nascent radiolabeled RNA was hybridized to control DNAs (β-actin and pBR322) and to test DNAs (ISG15 and ISG54) fixed to nitrocellulose.

Protein Analysis. Nuclear and cytoplasmic cell extracts were prepared as described and analyzed by electrophoretic mobility-shift assays of an end-labeled ISG15 oligonucleotide to quantitate ISGF3 (15, 17). The ISRE sequence contained within the oligonucleotide is in boldface type:

5′–GATCGGGAAA**GGGAAACCGAAACTGAA**GCC–3′
3′–CCCTTTCCCT**TTGGCTTTGACTTCGGCTAG**–5′.

An oligonucleotide of the inner core of the ISRE was also used:

5′–GATCCAAACCGAAAGG–3′
3′–GTTTGGCTTTCCCTAG–5′.

In vitro reconstitution of ISGF3 (15) was performed with a nuclear extract of IFN-α-stimulated cells that had been treated with 10 mM *N*-ethylmaleimide (NEM) for 10 min and quenched with 15 mM dithiothreitol to inactivate ISGF3γ and to provide a source of ISGF3α. The source of ISGF3γ was

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Abbreviations: IFN, interferon; ISG, IFN-stimulated gene; ISRE, IFN-stimulated response element; ISGF, IFN-stimulated gene factor; NEM, *N*-ethylmaleimide; SH, src homology.

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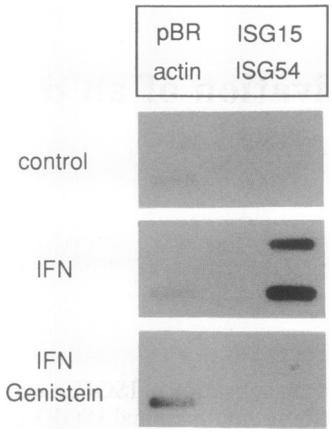

FIG. 1. Inhibition of tyrosine kinase activity blocks IFN-α stimulation of gene transcription. Nuclei were isolated from untreated cells (control) (*Top*) or cells stimulated with IFN-α in the presence (*Bottom*) or absence (*Middle*) of genistein. Nascent RNA was elongated *in vitro* in the presence of [^{32}P]UTP and hybridized to DNAs fixed to nitrocellulose. The positions of control plasmid pBR322, β-actin, and the IFN-α-stimulated genes ISG15 and ISG54 are indicated.

from a cytoplasmic extract of untreated cells. Ten micrograms of an ISGF3α-containing extract or 15 μg of an ISGF3γ-containing extract was mixed *in vitro* with 15 μg of the test cytoplasmic extract and subjected to mobility-shift electrophoresis.

Partial purification of ISGF3 (16) was achieved with a nuclear extract from HeLa cells treated overnight with IFN-γ and for 1 hr with IFN-α. After a 50% ammonium sulfate precipitation, protein was bound to Whatman P-11 phosphocellulose and eluted in 0.3 M KCl. This fraction was reequilibrated to 0.075 M KCl and applied to a nonspecific calf thymus DNA column (Sigma). ISGF3 was eluted in the 0.3 M KCl fraction, reequilibrated, and applied to a specific ISRE-Sepharose column (Pharmacia) (25). ISGF3 was eluted in 0.3 M KCl to obtain approximately 2000-fold purification.

Phosphotyrosine Immunoanalysis. Monoclonal antibodies to phosphotyrosine were obtained from Upstate Biotechnology (Lake Placid, NY) (4G10) or from ICN (PY20). For immunoblots, proteins were electroblotted from SDS/PAGE gels onto Immobilon P (Millipore) or nitrocellulose. The phosphotyrosyl-containing proteins were detected with a secondary antibody conjugated to horseradish peroxidase in an enhanced chemiluminescence reaction (Amersham). Purified control monoclonal antibody to a 92-kDa metalloproteinase was a gift of Deborah French (State University of New York at Stony Brook).

RESULTS

Tyrosine Kinase Activity Is Required for the Transcriptional Response to IFN-α. IFN-α induces the rapid transcription of a distinct set of genes. To determine if the activity of a tyrosine kinase is involved in the signal transduction pathway of IFN-α, the effect of genistein, a specific inhibitor of tyrosine kinases, was investigated. At the concentrations used, genistein inhibits the tyrosine kinase activity of the epidermal growth factor receptor, v-src, and p110$^{gag\text{-}fes}$, but does not have an effect on protein kinase C, protein kinase A, or phosphorylase kinase (26).

Nuclear run-on experiments were used to evaluate the transcriptional response of two IFN-α-stimulated genes, ISG15 and ISG54 (7, 9) (Fig. 1). Nuclei were isolated from untreated cells or from cells treated with IFN-α or IFN-α/genistein, and nascent RNA was elongated *in vitro* in the presence of [^{32}P]UTP. Hybridization of the radiolabeled RNA to specific genes clearly demonstrated that genistein blocked IFN-α-stimulated transcription.

Activation of the ISGF3α Subunit of ISGF3 Is Dependent upon Tyrosine Phosphorylation. IFN-α induces the activation of a latent transcription factor, ISGF3, that can bind to the ISRE. The effect of genistein on the activation of ISGF3 by IFN-α was investigated by electrophoretic mobility-shift analysis of the ISRE. Nuclear extracts were prepared from control cells and cells that were treated with IFN-α for 30 min with or without pretreatment with genistein. Nuclear protein extracts were subjected to gel mobility-shift electrophoresis with the ISRE (Fig. 2 *Left*). Pretreatment of cells with genistein inhibited the ability of IFN-α to induce the appearance of ISGF3 in the nucleus. To ensure that this inhibition was not merely a block in nuclear translocation, cytoplasmic fractions of the cellular lysates were also tested (Fig. 2 *Center*). Both the nuclear and cytoplasmic extracts from genistein-pretreated cells were deficient in ISGF3 ISRE-binding activity.

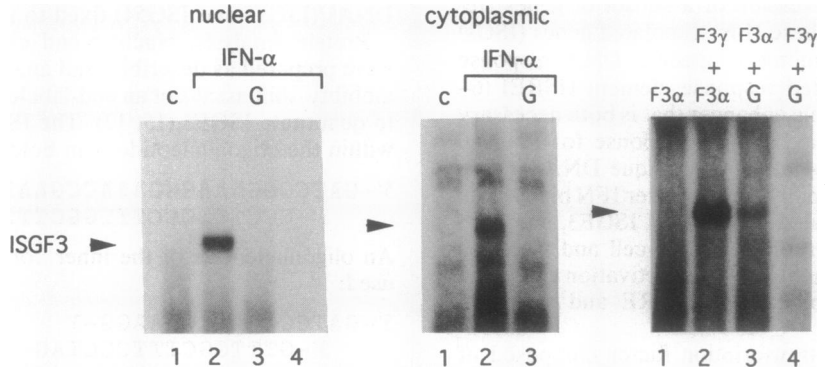

FIG. 2. Effect of genistein on the activation of ISGF3. Electrophoretic mobility-shift assays were performed with cell extracts and radiolabeled ISRE oligonucleotide. (*Left*) Nuclear extracts were prepared from control cells (c) (lane 1), or cells treated with IFN-α for 30 min in the absence (lane 2) or the presence (lane 3) of genistein (lanes G). Protein extract (5 μg) was used in the DNA-binding analyses with 1 ng of oligonucleotide. Specificity of the shifted complex is shown by competition with a 100-fold excess of unlabeled ISRE oligonucleotide (lane 4). (*Center*) Cytoplasmic extracts were prepared from control cells (c) (lane 1), or cells treated with IFN-α in the absence (lane 2) or presence (lane 3) of genistein (lanes G). Cytoplasmic protein (15 μg) was used in the assay. (*Right*) Reconstitution of ISGF3 *in vitro*. Protein extracts containing a source of ISGF3α (F3α) were prepared by NEM treatment and do not possess ISGF3 activity (lane 1). However, when mixed with a cytoplasmic extract from untreated cells to provide a source of ISGF3γ (F3γ), ISGF3 is reconstituted (lane 2). By mixing ISGF3α with a cytoplasmic extract from cells treated with IFN-α in the presence of genistein (G), ISGF3 is reconstituted (lane 3). However, mixing of the same cytoplasmic extract from IFN-α/genistein-treated cells (G) with a source of ISGF3γ did not reconstitute ISGF3 (lane 4).

To determine if genistein blocked the activity of one or both of the ISGF3 subunits, an established *in vitro* reconstitution assay was utilized (15) (Fig. 2 *Right*). *In vitro* combination of extracts containing ISGF3γ with extracts containing ISGF3α reconstitutes the ISGF3 functional complex (lane 2). The ISGF3γ subunit, but not the ISGF3α subunit, can be inactivated by treatment of ISGF3-containing extracts with NEM. Therefore, an NEM-treated nuclear extract from IFN-α-stimulated cells provided a source of ISGF3α (lane 1). Cytoplasmic extract from untreated cells contains ISGF3γ, but no ISGF3α, and served as a source of ISGF3γ. To test the effects of genistein on the individual components of ISGF3, cytoplasmic extract from genistein/IFN-α-treated cells was mixed *in vitro* with either ISGF3α (lane 3) or with ISGF3γ (lane 4). ISGF3 was reconstituted from genistein-treated extracts only upon addition of ISGF3α. Although the cytoplasmic level of ISGF3γ subunit appeared to be lower in genistein-treated extracts than untreated extracts, it was sufficient for *in vitro* reconstitution with ISGF3α. Therefore, genistein specifically blocked activation of the ISGF3α subunit.

ISGF3α Is Phosphorylated on Tyrosine Residues. Since cytoplasmic activation of ISGF3 requires the function of a tyrosine kinase, ISGF3 could serve as a substrate for a membrane-associated or cytosolic tyrosine kinase. To test for the presence of phosphotyrosine in activated ISGF3, we used an electrophoretic mobility supershift assay with a monoclonal antibody that recognizes phosphotyrosine (Fig. 3 *Left*). The source of ISGF3 was from an ISRE-affinity-purified preparation and contained ISGF3 as well as the free ISGF3γ subunit. Specific competition with the entire wild-type ISRE (wt) but not the central core sequence (core) verified the identity of these complexes in the purified extract (lanes 2 and 3) (13, 17). Monoclonal antibody to phosphotyrosine was included in the ISRE-binding reaction to test its effect on ISGF3–DNA complex formation. Inclusion of the anti-phosphotyrosine antibody produced a new supershift complex of the ISGF3-ISRE (lane 4), whereas control antibody had no effect (lane 5). The supershifted ISGF3 complex can be produced with three different anti-phosphotyrosine antibodies tested (4G10, PY20, and polyclonal), but is not produced with a variety of control antibodies. The supershift complex is a strong indication that the antibody to phosphotyrosine is bound to ISGF3 and, as a consequence, this larger protein–DNA complex migrates more slowly during electrophoresis. The percentage of ISGF3 that can be supershifted varies between 10% and 80% in individual experiments as shown (Fig. 3 *Left Top* and *Bottom*). This variation may be due to differences in the phosphotyrosine content of ISGF3 from independent preparations. There is no indication that ISGF3γ contains phosphotyrosine, since the ISRE-binding activity of the ISGF3γ subunit was not altered.

To ensure that the antibody to phosphotyrosine was contained in the supershifted ISGF3 complex, protein–DNA complexes were electroblotted to nitrocellulose and incubated with ^{125}I-labeled protein A (Fig. 3 *Center*). The ^{125}I-labeled protein A bound specifically to the supershifted complex, indicating the presence of the reactive IgG antibody to phosphotyrosine. Free IgG migrates diffusely at the bottom of the gel as detected by reaction with the enhanced chemiluminescence system (data not shown).

To verify the presence of phosphotyrosine in ISGF3, an immunodepletion experiment was performed (Fig. 3 *Right*). Antibody to phosphotyrosine was incubated with the ISRE-affinity-purified ISGF3, and the immunocomplexes were removed from the solution by binding to protein A-Sepharose beads and centrifugation. A portion of the depleted supernatant was analyzed for ISGF3 content in an ISRE mobility-shift assay (lane 3), and the remainder was subjected to a second round of depletion (lane 4). Incubation of the sample

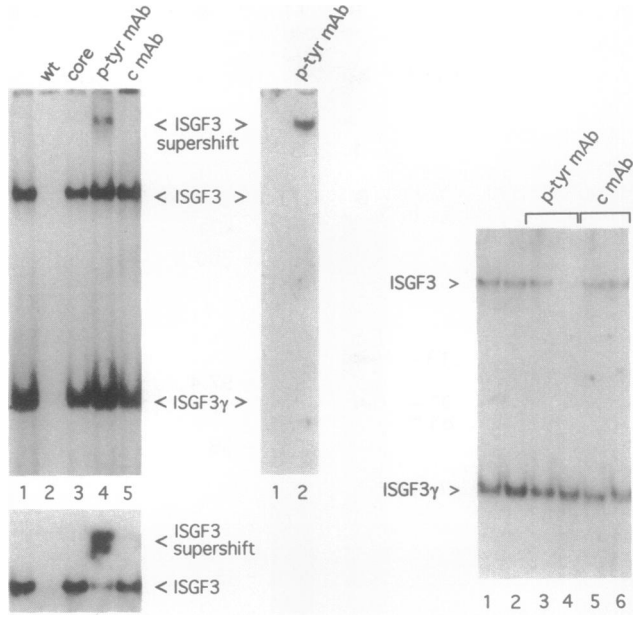

FIG. 3. ISGF3 is specifically recognized by an antibody to phosphotyrosine. (*Left Top*) ISRE-affinity purified ISGF3 was used in the gel mobility-shift analyses and reveals the presence of both ISGF3 and ISGF3γ (lane 1). Specific DNA-binding competition can be achieved with an excess of unlabeled ISRE probe (wt) (lane 2) but not with an oligonucleotide containing only the central nine nucleotides of the ISRE (core) (lane 3). Addition of 1 μg of anti-phosphotyrosine antibody (4G10) to the binding reaction prior to addition of the ISRE probe produces a supershifted complex of the ISGF3 (lane 4). Control antibodies have no effect (lane 5). (*Left Bottom*) A duplicate experiment displaying the variation in percentage of ISGF3 supershift. (*Center*) A mobility shift gel was performed with the ISGF3–ISRE in the absence of antibody (lane 1) or the presence of anti-phosphotyrosine antibody (lane 2). The proteins were electroblotted onto nitrocellulose and treated with ^{125}I-labeled protein A. (*Right*) Immunodepletion of ISGF3. The ISGF3 preparation was treated in the absence of antibody (lanes 1 and 2) or in the presence of anti-phosphotyrosine antibody (4G10) (lanes 3 and 4) or control antibody (lanes 5 and 6). The first binding reaction was performed for 1 hr on ice, and the immunocomplexes were removed with protein A-Sepharose. A portion of the supernatant fraction was analyzed by gel shift (lanes 1, 3, and 5). The remainder of the supernatant was used in a second depletion experiment (lanes 2, 4, and 6). The samples used in the gel shift were normalized for protein concentration to control for volume changes.

with the anti-phosphotyrosine antibody removed ISGF3 from the solution but did not remove the ISGF3γ subunit, which served as an internal control. Neither control antibody (lanes 5 and 6) nor protein A-Sepharose in the absence of antibody (lanes 1 and 2) depleted binding activity. This result indicates that a significant portion of the ISGF3 complex contains phosphotyrosine.

The ISGF3γ subunit of ISGF3 is a 48-kDa protein, whereas the ISGF3α subunit consists of three proteins of 113 kDa, 91 kDa, and 84 kDa (16, 17). To identify the specific proteins in ISGF3 that contain phosphotyrosine, an ISRE-affinity-purified preparation was analyzed by anti-phosphotyrosine immunoblotting (Fig. 4). Proteins that bound to the ISRE-Sepharose column (lane B) and proteins that flowed through the ISRE-Sepharose column (lane FT) were analyzed. A mobility-shift analysis of the bound and flow-through fractions is shown (Fig. 4 *Upper*). Proteins from these fractions were subjected to SDS/PAGE, electroblotted onto membrane, and treated with anti-phosphotyrosine antibody (Fig. 4 *Lower*). Three proteins that reacted with antibody to phosphotyrosine were identified only in the bound fraction of the ISRE oligonucleotide affinity column. These proteins

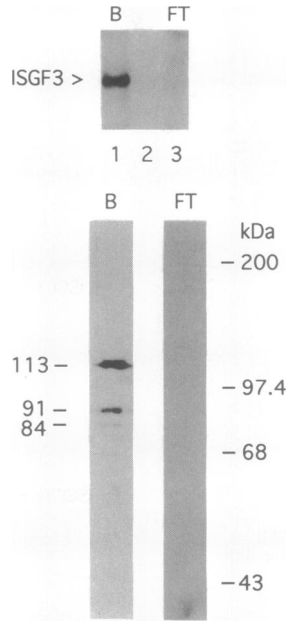

FIG. 4. Three proteins with mobilities of 113 kDa, 91 kDa, and 84 kDa copurify with ISGF3 and contain phosphotyrosine. (*Upper*) Mobility-shift analysis of partially purified ISGF3 from fractions that bound to the ISRE-affinity column (B) (lane 1) or proteins that flowed through the column (FT) (lane 3). Specific DNA competition is shown in lane 2. Samples were normalized for protein concentration. (*Lower*) Anti-phosphotyrosine immunoblots were performed with proteins that bound (B) to the ISRE-affinity column or flowed through (FT) the column. Approximately equal amounts of protein of the bound and flow-through fractions were dialyzed and lyophilized. Proteins were subjected to SDS/PAGE and immunoblotted with a mixture of PY20 and 4G10 anti-phosphotyrosine antibodies.

migrated at positions consistent with molecular masses of 113 kDa, 91 kDa, and 84 kDa, components of the ISGF3α subunit. These results strongly suggest that all three proteins composing the ISGF3α subunit are phosphorylated on tyrosine residues, but that the 48-kDa ISGF3γ subunit is not.

DISCUSSION

A working model of the IFN-α signal transduction pathway can be deduced from the data presented in this study and from data of previous studies referenced (Fig. 5). Although a gene encoding the receptor for IFN-α has been cloned (19), it has not provided an understanding of the IFN-α signal transduction mechanism. It remains unclear whether dimerization of the receptor or association with a distinct subunit is required (20, 21, 27). The activation of src-related tyrosine kinases has been demonstrated in a variety of signal transduction pathways (reviewed in refs. 27 and 28). The results presented in this study strongly suggest the participation of a tyrosine kinase(s) in the signal transduction pathway of IFN-α. A tyrosine kinase(s) may be associated with the IFN-α receptor and specifically activated upon IFN-α binding. Although a known or novel kinase may be responsible for this activity, it is of course possible that ISGF3 itself possesses tyrosine kinase activity. Previous studies have described the activation of protein kinase C isozymes after IFN-α binding (22, 29). The activation of protein kinase C may occur as a secondary consequence of activation of a phospholipase by tyrosine phosphorylation (30, 31).

Of foremost significance is the demonstration that ISGF3, a DNA-binding transcription factor, is a substrate for an IFN-α-activated tyrosine kinase(s). This result provides evidence for activation of a latent cytoplasmic transcription factor by tyrosine phosphorylation. ISGF3 is composed of

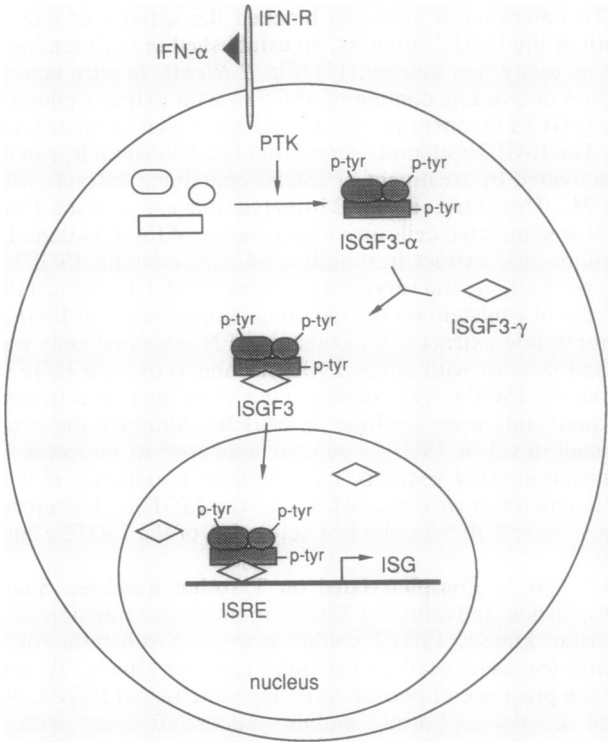

FIG. 5. Illustrative model of the IFN-α signal transduction pathway. IFN-α binds to its cell surface receptor (IFN-R), leading to the activation of a protein tyrosine kinase (PTK). The ISGF3α subunit proteins are substrates for the kinase. The activated ISGF3α subunit subsequently associates with the ISGF3γ subunit. The mature ISGF3 translocates to the nucleus, binds to the interferon-stimulated response element (ISRE), and induces transcription of interferon-stimulated genes (ISGs). Since the exact protein–protein interactions have not been delineated, the positions of the proteins in the complex are speculative.

two basic subunits, ISGF3α and ISGF3γ (15, 18). ISGF3α becomes a functional subunit in the cytoplasm after IFN-α stimulation. Three distinct proteins of 113 kDa, 91 kDa, and 84 kDa form the ISGF3α subunit (16, 17). The ISGF3γ subunit preexists in the cell as a 48-kDa protein and associates with ISGF3α to form the mature ISGF3. Although high levels of ISGF3γ are found in the nucleus, our model depicts association with the ISGF3α subunit in the cytoplasm. This model is based upon the observations that cytoplasts of enucleated cells form active ISGF3 in response to IFN (14) and that NaF treatment blocks translocation of ISGF3 to the nucleus but does not block cytoplasmic activation of ISGF3 (15). However, since ISGF3 complex formation is measured by a DNA-binding assay, it is possible that subunit association occurs on the ISRE. In either case, genistein acts to inhibit activation of the ISGF3α subunit of ISGF3 (Fig. 2).

Antibody to phosphotyrosine produces a supershift complex of the ISGF3–ISRE in electrophoretic mobility-shift analyses. Therefore, the ISGF3 that binds to DNA contains phosphotyrosine. In addition, the anti-phosphotyrosine immunoblot assays reveal three proteins containing phosphotyrosine that copurify with ISGF3 and are of a similar molecular weight as the ISGF3α subunit proteins. The 113-kDa and 91-kDa proteins consistently display the most reactivity with anti-phosphotyrosine antibodies. It is possible that the 84-kDa component contains less phosphotyrosine. Alternatively, the 84-kDa protein may not be present in stoichiometric amounts, since the protein composition of ISGF3 has only been estimated by silver staining of gels to date (16, 17). The immunoblot analyses suggest that all three proteins of the ISGF3α subunit are phosphorylated on tyro-

sine residues. This fact suggests the possibility of protein–protein interactions through src-homology (SH) protein domains (reviewed in refs. 32 and 33). SH2 domains have a binding affinity for peptide sequences containing phosphotyrosine. Tyrosine phosphorylation of ISGF3 component proteins may initiate complex formation and thereby the subsequent activation of a latent transcription factor. cDNAs have recently been isolated that correspond to the protein components of ISGF3 (34, 35). Analysis of the cDNA sequences may reveal putative functional domains of the proteins.

Tyrosine-phosphorylated ISGF3 serves as a messenger of a signal generated at the plasma membrane to the transcriptional machinery of the nucleus. It will be of interest to determine if tyrosine phosphorylation serves the predicted function in protein–protein association and/or in transcriptional stimulation.

Note. After this paper was submitted, articles by Fu (36) and Schindler *et al.* (37) reported the tyrosine phosphorylation of ISGF3α, and Fu predicted the presence of SH2 and SH3 domains in p113 and p91/84 from the cDNA sequences. A report by Velazquez *et al.* (38) demonstrated complementation of a mutant cell line unresponsive to IFN with a cosmid containing the Tyk2 tyrosine kinase gene.

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