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In Vitro Activation of the Transcription Factor ISGF3 by Interferon α Involves a Membrane-associated Tyrosine Phosphatase and Tyrosine Kinase*

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Interaction of interferon α (IFNα) with its cell surface receptor rapidly activates the formation of the transcription complex ISGF3, which subsequently translocates to the nucleus and stimulates the expression of a variety of early response genes. We have recently developed a cell-free system where IFNα can activate the formation of ISGF3 *in vitro*. This system has enabled us to demonstrate that the component of the ISGF3 transcription complex which is modified by IFNα treatment (ISGF3α) is membrane-associated and that its activation involves a protein kinase. Using a combination of specific tyrosine kinase and phosphatase inhibitors and monoclonal anti-phosphotyrosine antibodies we now are able to demonstrate that IFNα-activated transcription involves at least a two-step process where a membrane-associated tyrosine phosphatase and a tyrosine kinase lead to modification of ISGF3α and subsequent formation of the complete complex. Furthermore, formation of the ISGF3 complex is specifically disrupted by protein tyrosine phosphatase and can be reversibly dissociated by the phosphotyrosine analogue phenylphosphate. The latter observation suggested that SH2 and/or SH3 domains may be required for the stable formation of this transcription complex.

The diverse biological actions of interferons include protection against viral infection, inhibition of cell growth, and modulation of immune responses (1). In a manner similar to other cytokines and biological modifiers, interferons rapidly induce the expression of a set of early response genes, which presumably result in IFN's[1] biological effects. Cellular genes, which are activated by type I interferons (IFNα and β), contain within their promoters an interferon-stimulated response element (ISRE), which is necessary and sufficient to stimulate the transcription of these genes (2). Several proteins specifically interact with the ISRE, some of which display increased binding after treatment of cells with IFNα (2–4). One of these, ISGF3, is a multisubunit protein complex that is formed in the cytoplasm within minutes after treatment with IFNα. After translocating to the nucleus, it specifically binds to the ISRE and stimulates the transcription of IFNα-activated genes (2–4). The ISGF3 complex consists of four subunits, a 48-kDa protein (ISGF3γ), which binds to the ISRE alone, and three additional proteins of 84, 91, and 113 kDa (ISGF3α), which interact with ISGF3γ (5, 6). IFNα treatment of cells induces a modification of the ISGF3α proteins such that they can form a stable complex with ISGF3γ.

Although the IFNα receptor has been cloned (7), the signal transduction mechanisms by which IFNα activates gene expression or any of its other biologic activities still remains unclear. A number of reports have suggested that IFNα activates a protein kinase C, which subsequently can induce the expression of interferon-stimulated genes (ISGs) (8). However, other evidence suggests that this is not the case (9–11). In an attempt to elucidate the signaling mechanisms of these cytokines, we have recently developed a cell-free system where IFNα activates the formation of ISGF3 in HeLa cell homogenates (12). Activation of the ISGF3α proteins requires only a membrane-enriched fraction of HeLa cells. In addition, IFNα activation of ISGF3α requires ATP, but not guanine nucleotides or calcium, and is inhibited by high concentrations of staurosporin (12). These results implied that the signal transduction mechanism for activation of this transcription factor involves a protein kinase, but does not involve a G-protein-mediated coupling event or a calcium-dependent reaction. Based on these initial observations we have used this *in vitro* system to define a membrane-associated signaling cascade that is required for IFNα to activate this transcription factor.

MATERIALS AND METHODS

Preparation and Fractionation of HeLa Cell Homogenates—HeLa S3 cells from ATCC were grown in minimal essential spinner medium with 10% calf serum. To increase the endogenous concentration of ISGF3γ, HeLa cells (approximately 3×10^8) were incubated with recombinant human IFNγ (2.0 ng/ml) (Genentech) for 16 h. Cells were collected by centrifugation, washed with phosphate-buffered saline, and resuspended in 1.5 ml of reaction buffer (20 mM $MgCl_2$, 200 μM $CaCl_2$, 100 mM NaF, 100 mM Hepes (pH 7.9), 200 μM sodium ascorbate, 12 mM phosphoenolpyruvate, 4 mM ATP, 30 μg/ml pyruvate kinase, and 600 μM PMSF). Cells were homogenized, and portions (50 μl) of the lysate were incubated without or with recombinant

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[1] The abbreviations used are: IFN, interferon; ISRE, interferon-stimulated response element; EMSA, electrophoretic mobility shift assay; NEM, *N*-ethylmaleimide; PMSF, α-toluenesulfonyl fluoride; ISG, interferon-stimulated genes.

human IFNα-2a (Hoffmann LaRoche, 2×10^4 units) for the indicated times at 30 °C. Incubation was terminated by addition of 10 volumes of ice-cold stop solution (1 mM $MgCl_2$, 10 mM KCl, 20 mM Hepes (pH 7.0), 20% glycerol, 500 μM dithiothreitol, 250 μM PMSF, 0.1% Nonidet P-40). The mixture was vortexed and centrifuged at 18,000 × *g* for 5 min, and the supernatant was assayed for ISGF3 by EMSA with the ^{32}P-labeled oligonucleotide probe corresponding to the ISRE of ISG15.

For fractionation, homogenates from 3×10^8 cells were layered over a 39% sucrose solution and centrifuged for 5 min at 3000 × *g*. The upper phase was collected and centrifuged again at 18,000 × *g* for 5 min. The supernatant (SUP) was placed on ice, and the sedimented material was washed in 8 ml of 20 mM $MgCl_2$, 100 mM Hepes (pH 7.9), 200 μM sodium ascorbate, 600 μM PMSF, centrifuged again at 18,000 × *g* and resuspended in 2 ml of reaction buffer. Aliquots (50 μl) of membrane fraction were incubated with 10^3 units of IFNα at 30 °C, placed on ice, and Nonidet P-40 was added to a final concentration of 0.1%. The membrane fractions were vortexed and centrifuged at 18,000 × *g* for 5 min. The supernatant (10 μl) was mixed with 10 μl of SUP (diluted 1:10 with stop solution). The mixture was then assayed by EMSA.

Electrophoretic Mobility Shift Assays—Gel shift assays were performed essentially as described (3) using a ^{32}P-end-labeled synthetic oligonucleotide (1.0 ng) with the sequence (double-stranded) 5′-GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC-3′.

Treatment of ISGF 3 from HeLa Cell Homogenates with Tyrosine Phosphatase—HeLa cell homogenates were incubated with IFNα for 30 min as described. Recombinant tyrosine phosphatase isolated from *Yersinia* was expressed and purified as described (13).[2] Native or mutated forms of the enzyme (1 μg) were incubated 15 min at 30 °C with homogenates containing ISGF3 (50 μl) prior to EMSA using the ISRE probe.

Anti-phosphotyrosine Immunoprecipitation—Immunoprecipitations were performed using a monoclonal anti-phosphotyrosine antibody from Upstate Biotechnology. HeLa cell homogenates containing ISGF3 were incubated with 5 μg of antibody at 4 °C for 3–5 h. ISGF3 was then assayed by EMSA using the ISRE probe.

Anti-phosphotyrosine Immunoblotting—Immunoblotting was performed using a membrane-enriched fraction incubated with or without IFNα for 30 min at 30 °C. Nonidet P-40 was then added to 0.1%, and the membrane fractions were sedimented at 18,000 × *g* for 5 min. The supernatant was added to SDS sample buffer, and proteins were resolved by electrophoresis on 7% SDS-polyacrylamide gels and transferred to nitrocellulose. Western blotting was performed using a monoclonal anti-phosphotyrosine antibody from Upstate Biotechnology and a horseradish peroxidase-coupled secondary antibody (14). Immunoreactivity was detected by enhanced chemiluminescence (Amersham Corp).

RESULTS

IFNα Activation of ISGF3α in Membranes Is Inhibited by Genistein—We had previously determined that the ISGF3α proteins are membrane-associated and that their activation by IFNα requires ATP (12). We therefore decided to examine the effect of kinase inhibitors on activation of these proteins in a membrane-enriched fraction prepared from HeLa cells. These cells contain little of the DNA-binding protein ISGF3γ, which is necessary to form ISGF3. Because IFNγ increases the concentration of this protein (3), cells were incubated for 16 h with IFNγ prior to harvesting and preparation of a membrane-enriched fraction and a postnuclear supernatant. As shown previously (12), HeLa cells homogenized in reaction buffer and incubated for 30 min at 30 °C with IFNα displayed formation of ISGF3 when assayed by electrophoretic mobility shift using an ISRE probe (Fig. 1*A*, compare *lanes 1* and *2*). Neither the membrane nor supernatant fractions alone formed ISGF3 (*lanes 4–7*). When the membranes were first incubated with IFNα (30 °C for 30 min) and then mixed with supernatant (4 °C for 5 min), ISGF3 was reconstituted (compare *lanes 9* and *10*). ISGF3α, in contrast to ISGF3γ, is *N*-ethylmaleimide (NEM)-resistant. Membranes incubated with

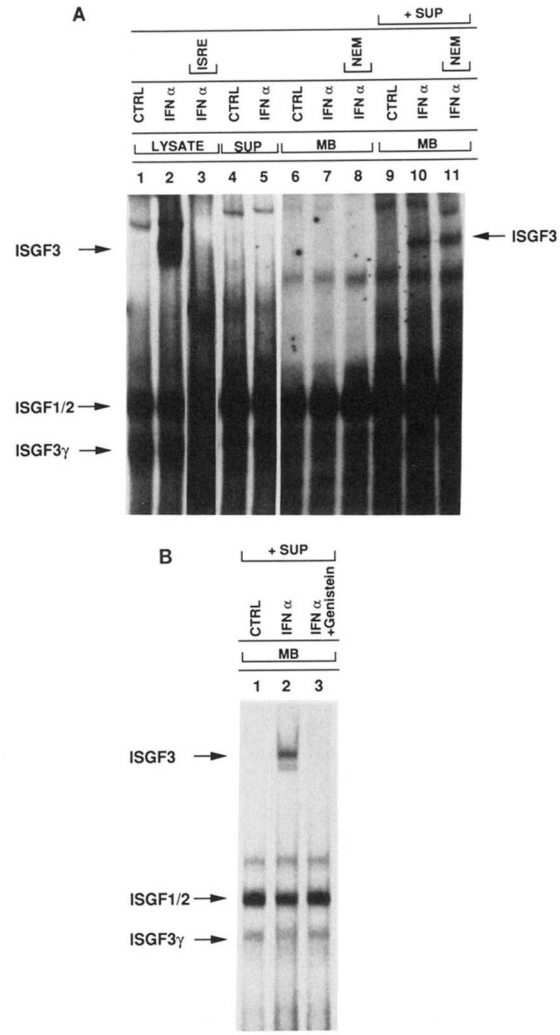

FIG. 1. *A*, activation of ISGF3 by IFNα in whole cell homogenates and after fractionation. Homogenates of HeLa cells were prepared as described under "Materials and Methods" and were incubated for 30 min at 30 °C without or with IFNα (*lanes 1* and *2*). The specificity of the ISGF3 complex was confirmed by competition with 50-fold molar excess of unlabeled probe (*lane 3*). After fractionation, the postnuclear supernatant (*SUP*) (*lanes 4* and *5*), and the sedimented membranes (*MB*) (*lanes 6–8*), were incubated separately without or with IFNα for 30 min at 30 °C, The membrane fraction was also treated with NEM after incubation with IFNα (*lane 11*). Addition of the supernatant fraction to the incubated membranes at 4 °C resulted in reconstitution of the ISGF3 complex only after IFNα activation of the membranes (compare *lanes 9* and *10*). NEM treatment of membranes after treatment with IFNα did not effect ISGF3 formation (*lane 11*). *B*, activation of ISGF3 in a membrane-enriched fraction by IFNα is inhibited by the tyrosine kinase inhibitor, genistein. A HeLa cell membrane fraction (*MB*) was incubated without (*lane 1*) or with IFNα (*lane 2*) for 30 min at 30 °C followed by addition of the supernatant fraction (*SUP*) at 4 °C. Incubation of the membranes with genistein (30 μg/ml) 10 min before IFNα was added inhibited the formation of ISGF3 (*lane 3*).

IFNα and subsequently treated with NEM and then mixed with supernatant at 4 °C also formed ISGF3 (*lane 11*). These results implied that: 1) activation of ISGF3 required incubation of only a membrane fraction with IFNα, 2) the supernatant fraction served as a source of ISGF3γ, and 3) some or all of the ISGF3α proteins (which are NEM-resistant) are membrane-associated and are modified such that they can interact with ISGF3γ.

The formation of ISGF3 was prevented both *in vivo* and *in vitro* by high concentrations of staurosporin, but at low con-

[2] Z.-Y. Zhang and J. E. Dixon, submitted for publication.

centrations of the drug, which specifically inhibit protein kinase C, no effect was seen (8, 9, 12). Since growth factors that act via tyrosine kinase-mediated signaling mechanisms often interact with plasma membrane-associated substrates (15), we used this cell-free system to examine the effects of genistein, a specific inhibitor of tyrosine kinases, on IFNα activation of ISGF3 (Fig. 1*B*). Incubation of a plasma membrane-enriched fraction prepared from HeLa cells with this agent 10 min prior to the addition of IFNα prevented formation of ISGF3 (Fig. 1*B*, compare *lanes 2* and *3*). However, addition of genistein after incubation of membranes with IFNα, but before mixing with the supernatant, had no effect (data not shown).

Assembly of the Subunits of the ISGF3 Transcription Complex Requires Phosphorylated Tyrosine—The results presented in Fig. 1 suggested that an IFNα-activated tyrosine kinase in a membrane-enriched fraction might be necessary to modify the ISGF3α protein(s) such that they can associate with each other and/or form a stable complex with ISGF3γ. If this hypothesis is correct, then it would be expected that anti-phosphotyrosine antibodies would interact with ISGF3. To examine this possibility, a monoclonal phosphotyrosine antibody was incubated with IFNα-treated lysates (Fig. 2*A*). After incubation of extracts with either phosphotyrosine-specific or nonspecific antibodies, gel shift assays were performed using an ISRE probe. The monoclonal anti-phosphotyrosine antibody specifically prevented the formation of the ISGF3 complex in a concentration-dependent manner, whereas incubation with control myeloma IgG2b immunoglobulin had little or no affect (*lanes 1* and *2 versus 3*). The binding of other proteins to the ISRE such as ISGF1 and ISGF2, which do not participate in the initial activation of ISGs by IFNα, was not affected by the anti-phosphotyrosine antibody. ISGF3γ binding to the ISRE in the absence of ISGF3α was also not disrupted by this antibody. Addition of the ISRE probe prior to the antibody prevented immunoprecipitation of ISGF3, suggesting that its interaction with DNA masked the phosphotyrosine residues recognized by the antibody (*lane 6*). Similar results to those shown in Fig. 2*A* were also seen with nuclear extracts prepared from human fibroblasts, and when other anti-phosphotyrosine antibodies were used (data not shown).

The use of anti-phosphotyrosine antibodies allowed us to determine that ISGF3α contained phosphorylated tyrosine residue(s). In order to evaluate if these residue(s) were necessary for assembly of this multisubunit complex, homogenates containing ISGF3 were incubated with purified recombinant protein tyrosine phosphatase from *Yersinia enterocolitica* (13).[2] This phosphatase has been found to specifically catalyze the removal of phosphate from tyrosine residues (13). It appears that protein tyrosine phosphatases require a catalytically essential cysteine residue for hydrolysis. Studies on the rat protein tyrosine phosphatase PTP1 have shown that this enzyme proceeds through a thiol phosphate intermediate (16). Incubation of IFNα-treated homogenates with this tyrosine phosphatase resulted in the loss of ISGF3 activity (Fig. 2*B*, compare *lanes 1* and *3*). As with the phosphotyrosine antibodies, incubation of extracts with purified tyrosine phosphatase did not significantly disrupt the binding of ISGF1/ISGF2 or ISGF3γ to the ISRE (these complexes are indicated in the figures). If extracts were incubated with the phosphatase in the presence of vanadate (a specific tyrosine phosphatase inhibitor), then ISGF3 activity was only slightly lost (*lane 4*). A recombinant phosphatase, which contains a cysteine to serine mutation in its active site and has no enzymatic activity (13),[2] was without effect on ISGF3 (*lane 2*).

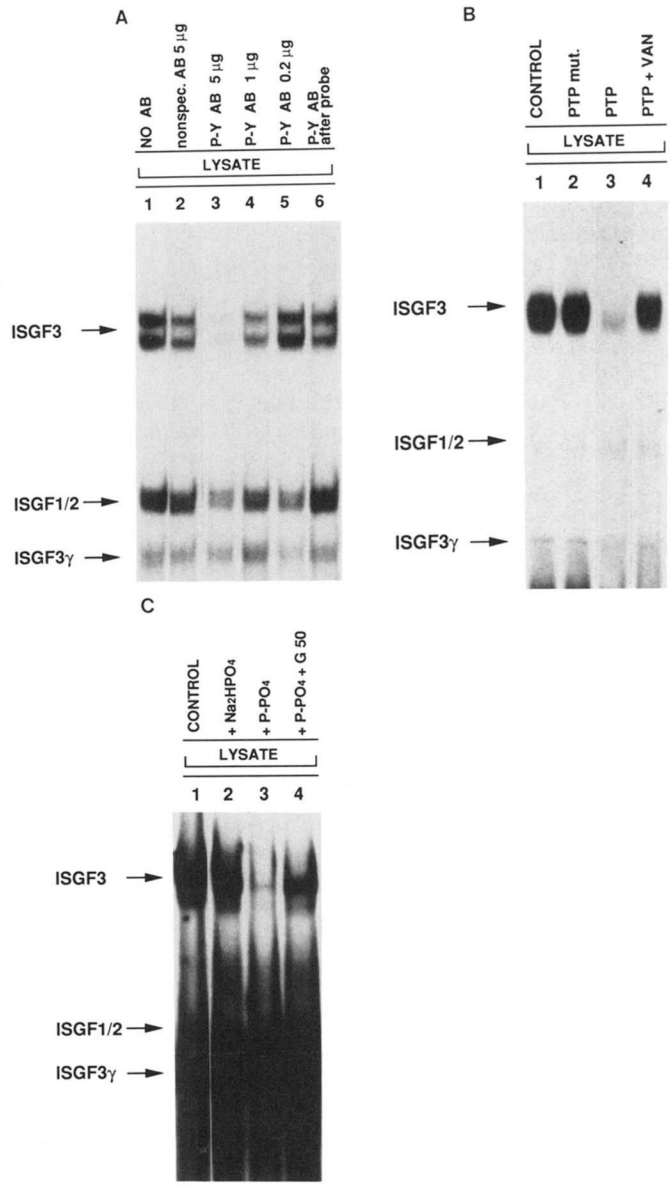

FIG. 2. **Phosphorylated tyrosine is necessary for assembly of the ISGF3 transcription complex.** *A*, anti-phosphotyrosine antibodies immunoprecipitated ISGF3. HeLa cell homogenates were prepared as described and were incubated with IFNα for 30 min as in Fig. 1*B*. Anti-phosphotyrosine antibodies were incubated with homogenates for 3 h at 4 °C, and products were assayed by gel shift. *Lane 1*, no addition; *lane 2*, incubation with a nonspecific antibody; *lanes 3–5*, as *lane 2*, but different concentrations of a monoclonal phosphotyrosine antibody were used; *lane 6*, as *lane 3*, but the probe was added before the antibody. *B*, recombinant tyrosine phosphatase prevents ISGF3 formation. ISGF3 formed in HeLa homogenates prepared as in *A* were incubated 30 °C for 15 min with nothing (*lane 1*), the mutant phosphatase (1 μg) (*lane 2*), the native enzyme (*lane 3*), or the native enzyme in the presence of 1 mM vanadate (*lane 4*). After incubation samples were subjected to EMSA using the ISRE probe. *C*, phenylphosphate reversibly dissociates ISGF3. HeLa cell homogenates were prepared as described above and subjected to EMSA (3). No addition (*lane 1*); 100 mM sodium phosphate (*lane 2*) or 50 mM phenylphosphate (*lane 3*) was added to the EMSA binding reaction which included the ISRE probe. *Lane 4* was the same as *lane 3* except the mixture was centrifuged over a Sephadex G50 spin column prior to loading on the gel.

The results obtained using the anti-phosphotyrosine antibodies and purified tyrosine phosphatase indicated that phosphorylated tyrosine in ISGF3α was needed for formation of the complex. To examine this possibility in greater detail,

experiments were performed to reversibly dissociate the complex with the phosphotyrosine analogue phenylphosphate. This compound has been widely used to disrupt associations between anti-phosphotyrosine antibodies and tyrosine-phosphorylated proteins. Lysates were prepared from HeLa cells, treated with IFNα, and gel shift assays were performed using the ISRE probe (Fig. 2*C*). IFGF3 from lysates was incubated with nothing (*lane 1*), phenylphosphate (*lane 3*), or sodium phosphate (*lane 2*) to control for any effects of salt concentration on ISGF3 formation. Phenylphosphate not only specifically disrupted ISGF3 (compare *lanes 1* and *3*), its removal from the binding reaction using a Sephadex G50 column permitted the ISGF3 complex to reassociate (*lane 4*).

Incubation of Only a Membrane Fraction with IFNα Induces Tyrosine Phosphorylation of Proteins with Molecular Sizes Corresponding to the ISGF3α Proteins—The results presented in Fig. 2 indicated that phosphorylated tyrosines are necessary to form the ISGF3 complex, and that dephosphorylation of these tyrosine residues did not affect the interaction of the ISGF3γ component with the ISRE. Genistein inhibited activation of the ISGF3α proteins (see Fig. 1*B*), suggesting that the IFNα-induced tyrosine phosphorylation of the ISGF3α protein(s) was necessary for their association with ISGF3γ. To examine which of the ISGF3α proteins were tyrosine-phosphorylated, Western blots were done using an anti-phosphotyrosine antibody as a probe. A membrane-enriched fraction was incubated with or without IFNα for 30 min. The membranes were then solubilized with the detergent Nonidet P-40 such that the ISGF3α proteins were extracted. Extracts were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose, and blots were probed with anti-phosphotyrosine antibody (Fig. 3). The same solubilized material was analyzed by gel shift to ensure that the membranes were IFNα-responsive in terms of activation of ISGF3 (data not shown). Two proteins were tyrosine-phosphorylated upon IFNα treatment of the membrane fraction (*lane 2*). These proteins corresponded in size to two of the ISGF3α proteins (113 and 91 kDa), which have been previously purified (5).

Evidence for an IFNα-stimulated Tyrosine Phosphatase Activity Required for ISGF3 Formation—Since it is becoming increasingly clear that cell surface receptor-activated signaling pathways that are coupled to tyrosine kinases may also require a tyrosine phosphatase activity, we examined the potential role of tyrosine phosphatases in activation of the ISGF3α proteins *in vitro*. Membrane-enriched fractions were incubated with the tyrosine phosphatase inhibitor vanadate 5 min before addition of IFNα for 30 min. After the incubation the membranes were mixed with the supernatant at 4 °C (Fig. 4*A*). Vanadate inhibited the formation of ISGF3 when added to the membranes before IFNα treatment (Fig. 4*A*, *lanes 1 versus 2* and *4 versus 5*), whereas addition of the serine/threonine phosphatase inhibitors okadaic acid or calyculin A had no effect (data not shown). However, if vanadate was added 5 min after IFNα, it was without effect (Fig. 4*A*, *lanes 1 versus 3* and *4 versus 6*). Since phenylphosphate reversibly dissociated ISGF3 (Fig. 2*C*), experiments were also done to determine whether it might have an inhibitory effect on activation of ISGF3. In a manner similar to vanadate, phenylphosphate prevented IFNα activation of ISGF3 when added 5 min prior to the cytokine (Fig. 4*B*, compare *lanes 3* and *5*), but not when it was added 5 min after IFNα (compare *lanes 3* and *7*). Removal of phenylphosphate from the reaction preincubated with the agent (*lane 5*) with the use of a G50 Sephadex column did not reconstitute ISGF3 binding (data not shown). Interestingly, it required only 10 mM phenylphosphate to inhibit the signaling in membranes, whereas it required 50 mM of this agent to dissociate ISGF3 (see Fig. 2*C*). Thus the addition of 10 mM phenylphosphate after 30 min of IFNα treatment of membranes had no effect on the transcription complex (compare *lanes 8* and *9*). This time-dependent effect of vanadate and phenylphosphate on inhibition of ISGF3α production contrasts with the inhibitory effects of genistein, which blocked further activation of ISGF3α at any time that it was added. Fig. 5 shows the results of addition of either vanadate (Fig. 5*A*) or genistein (Fig. 5*B*) to membranes before or after the addition of IFNα. ISGF3 formation as analyzed by gel shift was then quantitated directly by counting. These results not only implicated a tyrosine kinase in this signaling mechanism, but also suggested that an IFNα-stimulated tyrosine phosphatase was activated prior to the kinase since the phosphatase activity was not needed for the continued activation of ISGF3α by IFNα.

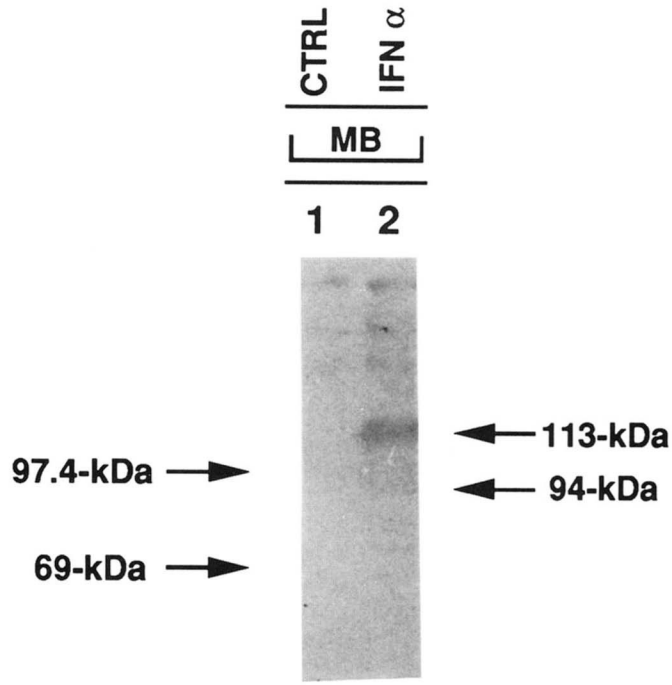

FIG. 3. **Incubation of a plasma membrane-enriched fraction of HeLa cells with IFNα induces the phosphorylation of tyrosine in proteins of 113 and 94 kDa.** A membrane fraction of HeLa cells was prepared as in Fig. 1*B* was incubated 30 °C for 30 min without (*lane 1*) or with IFNα (*lane 2*). Nonidet P-40 (0.1%) was added to the reaction, the membranes were vortexed and sedimented, and SDS sample buffer was added to the supernatant. Proteins were resolved on 7% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with an anti-phosphotyrosine antibody. The molecular size standards are indicated by the *arrows* on the *left*. The specific tyrosine phosphorylated species and their molecular sizes are shown by the *arrows* on the *right*.

DISCUSSION

Elucidation of the mechanisms involved in interferon α signaling of cells has been hampered by the absence of a system capable of yielding conclusive results. Although several investigators have taken advantage of the ability of IFNα to rapidly induce the formation of ISGF3 *in vivo*, these studies have the shortcomings of any investigation utilizing toxic inhibitors in an intact cell (8, 17, 18). To circumvent this problem, as well as to begin purification of the signaling components necessary for IFNα-induced gene expression, we have recently designed a cell-free system where IFNα can activate the formation of ISGF3 in a manner similar to that seen *in vivo* (12). Using this *in vitro* system, it has been

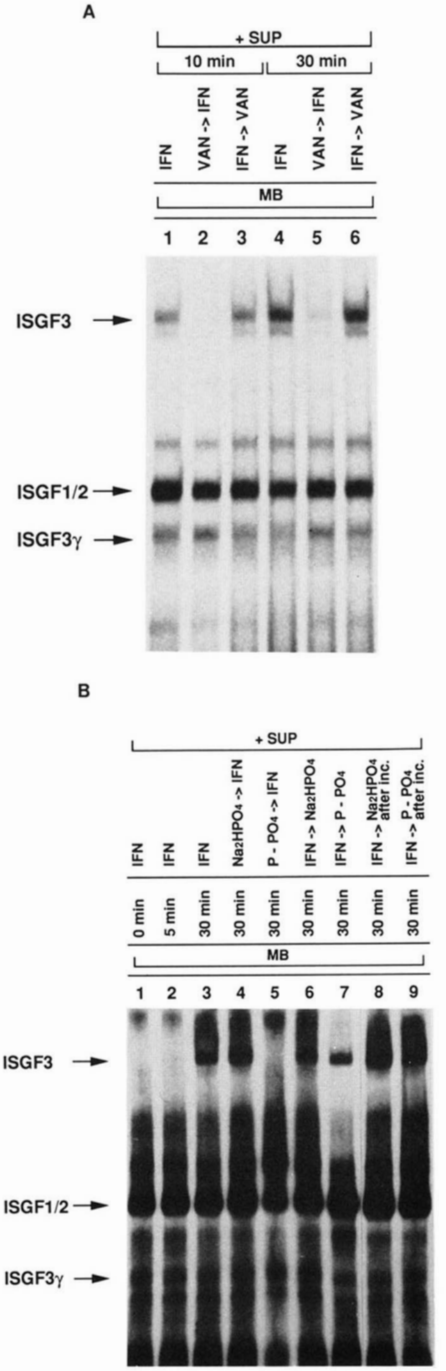

FIG. 4. **Activation of ISGF3 by IFNα in plasma membranes is inhibited by vanadate or phenylphosphate.** *A*, a plasma membrane-enriched fraction of HeLa cells was incubated at 30 °C with IFNα for either 10 min (*lanes 1–3*) or 30 min (*lanes 4–6*). Vanadate (1 mM) was added to some samples for 5 min before addition of IFNα (*lanes 2* and *5*) or 5 min after IFNα (*lanes 3* and *6*). After incubation the membrane fractions (*MB*) were mixed with the supernatant (*SUP*) fraction as in Fig. 1*B*, and analyzed by EMSA. *B*, essentially the same experiment as in *A* was performed, except that phenylphosphate was used. *Lane 1*, IFNα 0 min; *lane 2*, IFNα 5 min; *lane 3*, IFNα 30 min; *lane 4*, sodium phosphate (10 mM, pH 7.4) added 5 min prior to the addition of IFNα for 30 min; *lane 5*, same as *lane 4* except that phenylphosphate (10 mM) was used; *lane 6*, same as *lane 3* but sodium phosphate was added 5 min after IFNα; *lane 7*, same as *lane 5* except that phenylphosphate was added 5 min after IFNα; *lane 8*, same as *lane 3* but sodium phosphate was added after incubation with IFNα for 30 min; *lane 9*, same as *lane 5* but phenylphosphate was added after incubation with IFNα for 30 min.

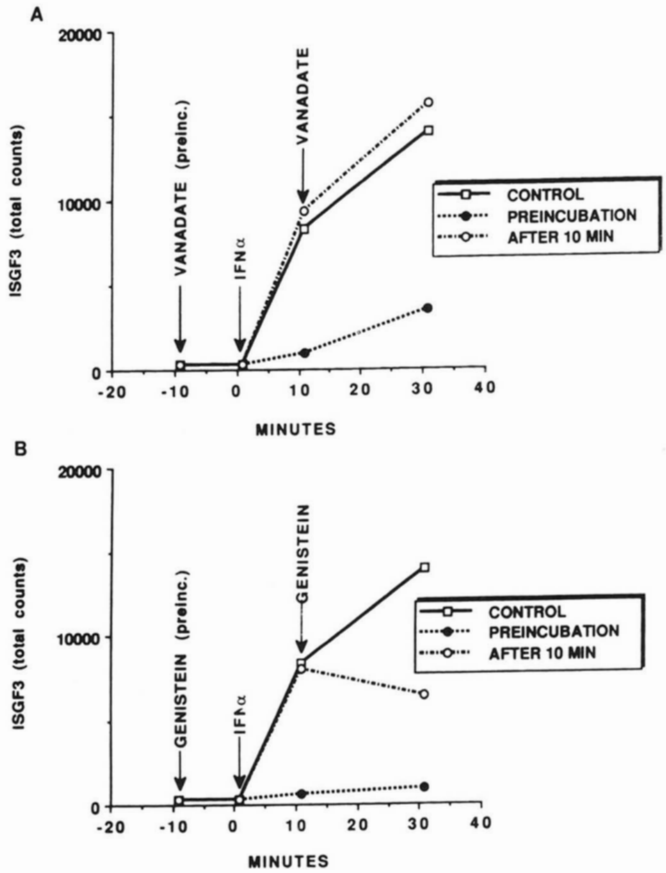

FIG. 5. **Quantitation of ISGF3 production in membrane fractions incubated with either genistein or vanadate.** Aliquots of a membrane-enriched fraction of HeLa cells were incubated with either vanadate (1 mM) (*A*) or genistein (30 μg/ml) (*B*). These inhibitors were added either 10 min before or 10 min after IFNα. Incubation with IFNα was terminated after 10 or 30 min, and ISGF3 was reconstituted using the supernatant fraction as described in Fig. 1*B*. After EMSA using the ISRE probe, ISGF3 formation was determined by direct counting (Ambis Systems). The *ordinate* in each *graph* represents total counts incorporated into the ISGF3 complex after a 12-h scan.

determined that the ISGF3α proteins, which are directly modified by IFNα, are probably associated with the plasma membrane (see Figs. 1 and 3). Activation of these proteins by IFNα occurs on the membrane followed by a release step that as yet remains to be defined. Genistein, a highly specific inhibitor of tyrosine kinases (19) blocked IFNα activation of ISGF3α in a membrane fraction (Fig. 1*C*). Addition of genistein after incubation of membranes with IFNα, but before reconstitution of ISGF3 with the supernatant was without effect. To confirm that ISGF3 contained phosphotyrosine, the transcription complex was immunoprecipitated with anti-phosphotyrosine antibodies. Although it has been previously shown that ISGF3 could be disrupted by treatment with alkaline phosphatase (20), which removes phosphate from serine, threonine, and tyrosine residues, we were able to extend this observation to show that tyrosine phosphatase specifically dissociated the complex (Fig. 2*B*). Furthermore, the phosphotyrosine analogue phenylphosphate could reversible dissociate ISGF3 (Fig. 2*C*). Since neither the anti-phosphotyrosine antibodies, tyrosine phosphatase, nor phenylphosphate disrupted the binding of ISGF3γ to the ISRE, it appeared that the ISGF3α proteins require tyrosine phosphorylation to either associate with themselves and/or interact with ISGF3γ. Whether all the ISGF3α proteins become

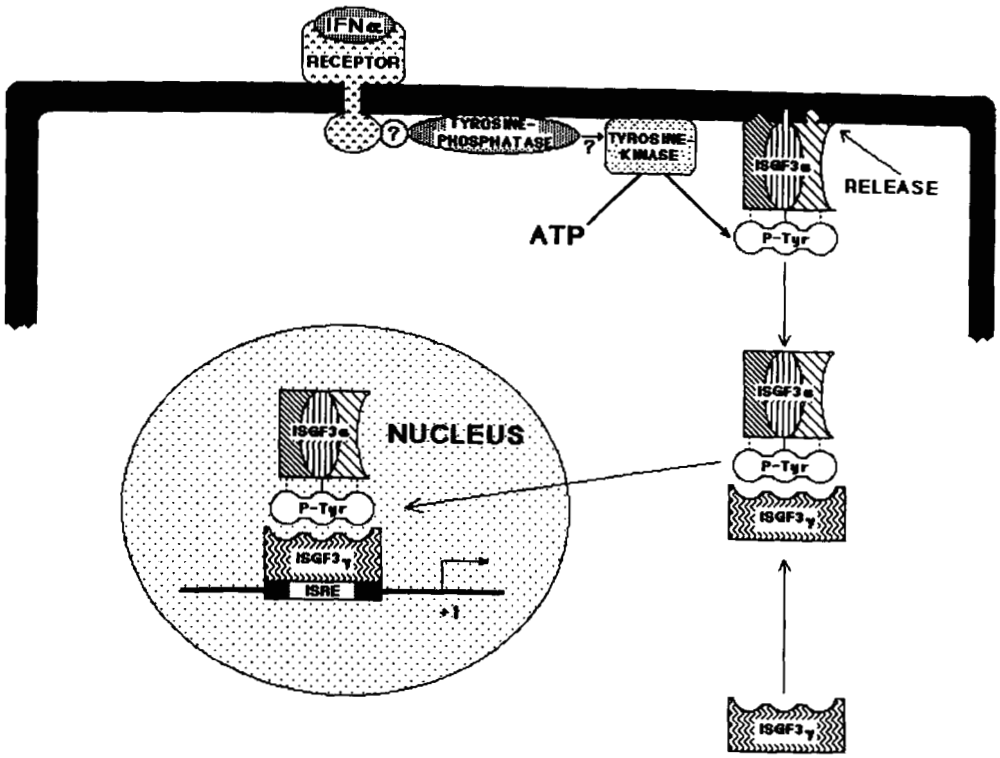

FIG. 6. **Representation of IFNα activation of ISGF3.** Binding of IFNα to its plasma membrane receptor directly or indirectly activates a protein tyrosine phosphatase, which leads to the activation of a tyrosine kinase. The tyrosine kinase catalyzes the tyrosine phosphorylation of some or all of the ISGF3α proteins. ISGF3α is then released from the plasma membrane into the cytosol, where it can interact with ISGF3γ and form ISGF3.

tyrosine-phosphorylated after IFNα treatment remains unclear. Western blots using anti-phosphotyrosine antibodies as a probe suggested that at least the 113-kDa protein and, to a lesser degree, the 91-kDa protein are probably tyrosine-phosphorylated as a result of incubation of membranes with IFNα (Fig. 3). Recent reports (10, 21) confirm these findings and indicated that all three ISGF3α proteins are tyrosine-phosphorylated with treatment of cells with IFNα.

Vanadate inhibition of activation of ISGF3 by IFNα suggested the role of a tyrosine phosphatase in the signaling process (see Figs. 4*A* and 5). This conclusion was reinforced by the specific kinetic patterns observed in the presence of the inhibitor. Vanadate was effective only when incubated with membranes before the addition of IFNα, but addition of the inhibitor 5 min after the cytokine was without effect. Likewise the addition of phenylphosphate also inhibited activation of ISGF3α only when added to the membrane fraction prior to the addition of IFNα (Fig. 4*B*). This inhibitory effect of phenylphosphate should not be confused with disruption of the ISGF3 complex as the latter required a much higher concentration of the inhibitor (at least 3-fold greater).

The precise role in this putative tyrosine phosphatase in the signaling process is not yet clear. One possible mechanism is the activation of a specific tyrosine kinase by dephosphorylation of one or more tyrosine residues, in a manner analogous to that described for the activation of the T-cell membrane-associated tyrosine kinase $pp56^{lck}$ by the tyrosine phosphatase CD45 (22, 23). Like other members of the Src family of protein kinases, dephosphorylation of a single tyrosine residue is necessary for $pp56^{lck}$ activity (24, 25). A second potential mechanism involves the removal of specific tyrosine phosphate(s) from ISGF3α followed by phosphorylation of other residues on the proteins by a tyrosine kinase, which may be either one of the ISGF3α proteins or a different species. However, since no phosphorylated tyrosines were detected on proteins with molecular sizes similar to ISGF3α in membranes before exposure to IFNα, and others have not observed constitutive phosphotyrosine in the ISGF3α proteins (10, 21), this possibility is unlikely (Fig. 3). The simplest and most conservative mechanism to interpret our data is shown in Fig. 6. IFNα is proposed to activate a tyrosine phosphatase, which in turn stimulates a tyrosine kinase by dephosphorylation of specific tyrosine residues. Once the kinase is activated, further phosphatase activity is not required. Following its activation by the tyrosine phosphatase, the putative kinase phosphorylates specific tyrosine residues on the ISGF3α peptides, which then can form a stable complex, leave the membrane, bind ISGF3γ, and translocate to the nucleus. This hypothesis is reinforced by the recent cloning of a tyrosine kinase (IFN-tyk) that complements a cell line that is unresponsive to IFNα (11). IFN-tyk is a member of the Src family of tyrosine kinases and, interestingly, contains a domain in its carboxyl-terminal region that is highly analagous to $pp60^{src}$. A phosphorylated tyrosine in this region of Src maintains the enzyme in an inactive state (15, 26).

The role of tyrosine phosphorylation has been well established in several signaling systems (15). The most commonly found structural motifs in tyrosine kinase substrates are the Src homology 2 and 3 (SH2 and SH3) domains in proteins associated with signal transduction systems, such as phospholipase C-γ, *ras*-GAP and the phosphatidylinositol 3′-kinase-associated pp85 (15). SH2 domains have also been recently described in tyrosine phosphatases (27). We have demonstrated in this work that tyrosine phosphorylation is required for the formation of the active ISGF3 complex. It is thus tempting to speculate that these structural domains may

participate directly in the formation of the active complex. The fact that phenylphosphate disrupted both the formation of ISGF3 and the activation of ISGF3α in membranes is supportive of this idea, and indeed a recent report has indicated that the ISGF3α proteins contain conserved SH2 and SH3 domains (10). However, whether the membrane-associated tyrosine phosphatase and kinase contain SH2 and/or SH3 domains and understanding the spatial relationship between these enzymes and the IFNα receptor will require purification of this multicomponent signaling complex.

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