

- and protein A/G agarose (15  $\mu$ l) for 90 min at 4°C with agitation. Agarose beads were washed three times in cell lysis buffer and the bound proteins were eluted in SDS gel loading buffer. The proteins were resolved on a 7% polyacrylamide SDS gel and visualized by autoradiography.
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- gel. The gel slice was dried, swollen in 10% acetic acid–40% methanol, transferred to then 50% methanol, and redried. The gel slices were then rehydrated in 0.5 ml, 50 mM  $\text{NH}_4\text{CO}_3$ , 0.1 mg/ml thermolysin (Calbiochem), and digested for 18 to 20 hours at 37°C. The eluted peptides were then subjected to two-dimensional phosphopeptide analysis (32) (pH 3.5, electrophoresis followed by chromatography in 1-butanol:H<sub>2</sub>O:pyridine:acetic acids, 15:12:10:3).
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## Activation of Transcription Factors by Interferon-alpha in a Cell-Free System

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The signal transduction mechanisms of interferons (IFNs) remain unclear partly because no effect of IFN has been reproducible in a cell-free system. IFN- $\alpha$  rapidly induces the transcription of a set of early response genes, and a multicomponent transcriptional activator, interferon stimulated gene factor 3 (ISGF3), is activated within minutes after binding of IFN- $\alpha$  to its receptor. A system was developed in which IFN- $\alpha$  activated ISGF3 in homogenates of HeLa cells. Subcellular fractionation revealed that incubation of a plasma membrane-enriched fraction with IFN- $\alpha$  was sufficient to activate the regulatory subcomponent of ISGF3.

Interferons are polypeptides that exhibit diverse biological activities such as inhibition of cell growth and inhibition of viral infection. Transcription of cellular genes is activated by type I interferons (IFN- $\alpha$  and IFN- $\beta$ ). The transcribed genes contain within their promoters similar sequences, termed interferon stimulated response elements (ISREs), which are necessary and sufficient for IFN- $\alpha$ -induced expression (1, 2). Two IFN- $\alpha$ -induced protein complexes (ISGF2 and ISGF3) and one constitutive protein complex (ISGF1) have been defined by means of electrophoretic mobility-shift gel assays (EMSA) with ISRE probes (1, 3, 4). ISGF3, a multisubunit protein complex, appears to be the primary positive regulator of IFN- $\alpha$ -induced gene transcription (1, 3, 4). The DNA binding component of the complex, ISGF3 $\gamma$ , has a molecular size of 48 kD and interacts directly with the ISRE (5). The amount of this protein is increased by prolonged treatment of cells with IFN- $\gamma$  (5, 6), and the constitutive concentration of ISGF3 $\gamma$  in the cell varies from one cell type to another. ISGF3 $\alpha$  consists of three proteins of 84, 91, and 113 kD, which are present in a latent form

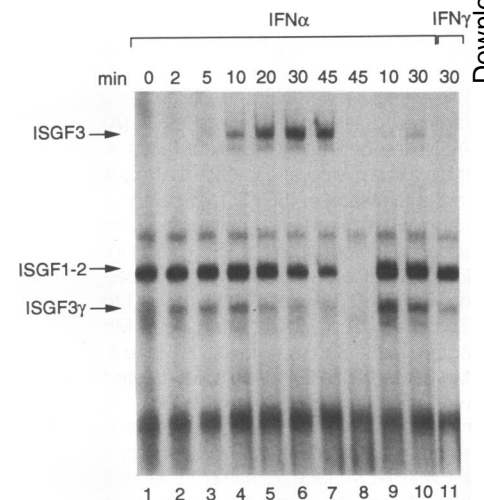
outside of the nucleus (7). The three proteins are rapidly modified when IFN- $\alpha$  binds to cells, and they then interact with ISGF3 $\gamma$  to activate IFN- $\alpha$ -stimulated genes (1, 7).

We prepared homogenates from HeLa

**Fig. 1.** Activation by IFN- $\alpha$  of formation of ISGF3 in homogenized HeLa cells. HeLa cells (approximately  $3 \times 10^8$ ) were incubated with recombinant human IFN- $\gamma$  (1 ng/ml) (Genentech) for 12 hours. Cells were collected by centrifugation, washed with phosphate-buffered saline, and resuspended in 1.5 ml of reaction buffer [20 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$   $\text{CaCl}_2$ , 100 mM NaF, 100 mM Hepes (pH 7.9), 200  $\mu\text{M}$  Na-ascorbate, 12 mM phosphoenolpyruvate, 4 mM ATP, 150  $\mu\text{M}$  GTP- $\gamma$ -S, pyruvate kinase (30  $\mu\text{g}/\text{ml}$ ), and 600  $\mu\text{M}$  PMSF]. Cells were homogenized and portions (50  $\mu\text{l}$ ) of the lysate were incubated without or with recombinant human IFN- $\alpha$ -2a (Hoffmann-La Roche,  $2 \times 10^4$  units) for the indicated times at 30°C. Incubation was terminated by addition of ice-cold stop solution [1 mM  $\text{MgCl}_2$ , 10 mM KCl, 20 mM Hepes (pH 7.0), 20% glycerol, 500  $\mu\text{M}$  dithiothreitol (DTT), 250  $\mu\text{M}$  PMSF, 0.1% NP-40]. The mixture was centrifuged at 18,000g and the supernatant was assayed for ISGF3 (3) by EMSA with a <sup>32</sup>P-labeled oligonucleotide probe corresponding to the ISRE of ISG15 (5'-GATCCATGCCCTCGGAAAGG-GAAACCGAAACTGAAGCC-3') and its complement. Lanes 1 to 7, time course of IFN- $\alpha$ -induced ISGF3 formation; lane 8, same as lane 7 but except that a 50-fold molar excess of unlabeled ISRE was added to the binding reaction; lanes 9 and 10, incubation of homogenates with IFN- $\alpha$  but without ATP and pyruvate kinase (the band in lane 10 may form because there is endogenous ATP in the lysate); lane 11, incubation with IFN- $\gamma$  (50 ng/ml) for 30 min.

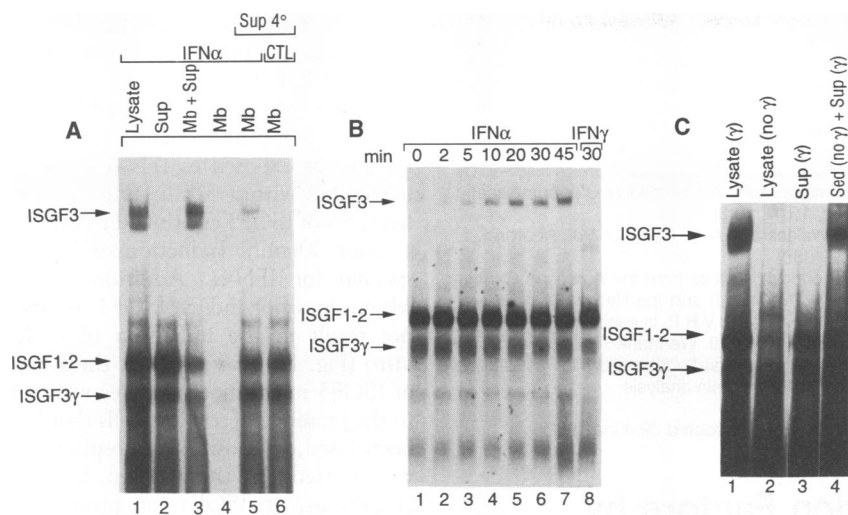
cells that had been exposed to IFN- $\gamma$  for 8 to 12 hours to induce sufficient concentrations of ISGF3 $\gamma$  so that IFN- $\alpha$  activation of ISGF3 $\alpha$  could be detected by EMSA. IFN- $\alpha$ -induced ISGF3 formation in HeLa homogenates exposed to IFN- $\alpha$  at 30°C was detectable within 5 min (Fig. 1) and the amounts of ISGF3 continued to increase for at least 30 min. Induction of ISGF3 was specific for IFN- $\alpha$ . Addition of IFN- $\gamma$ , which does not induce ISGF3 in vivo, did not result in the induction of ISGF3 in vitro (Fig. 1). To verify that the activation of ISGF3 in the homogenates was not due to the presence of residual cells that had not been lysed, adenosine triphosphate (ATP) was omitted from the reaction. Under these conditions, no ISGF3 was produced (Fig. 1). The nonhydrolyzable ATP analogue adenylyl-( $\beta$ , $\gamma$ -methylene)-diphosphonate (AMP-PCP) did not substitute for ATP (8). The ISGF3 complex co-migrated with ISGF3 formed in intact cells exposed to IFN- $\alpha$  (8).

To obtain more information concerning IFN- $\alpha$  activation of ISGF3, HeLa cell homogenates were fractionated. We took advantage of the fact that ISGF3 $\gamma$ , the DNA-binding component of ISGF3, is not modified in cells treated with IFN- $\alpha$  and is present in both the cytoplasm and the nucleus (1, 6). Nuclei were separated from cytoplasm and membranes by centrifugation through a layer of 39% sucrose at 2500g. The remaining cellular material was then centrifuged at 18,000g. This yielded two components, which we refer to as the supernatant (Sup) and the sedimented crude membrane (Mb) fraction. The Mb fraction was washed, centrifuged again, and



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**Fig. 2.** Characterization of HeLa cell homogenate fractions required for IFN- $\alpha$ -induced formation of ISGF3. **(A)** Requirement of a HeLa cell fraction that contains plasma membranes for IFN- $\alpha$ -induced formation of ISGF3. HeLa cells were incubated with IFN- $\gamma$  for 12 hours. A homogenate was prepared as in Fig. 1, and a portion was incubated with IFN- $\alpha$  for 30 min (lane 1). The remaining homogenate was layered over a 39% sucrose solution and centrifuged for 5 min at 2500g. The upper layer was collected and centrifuged again at 15,000g for 5 min. The supernatant (Sup) was placed on ice, and the sedimented material (Mb) was washed in 20 mM MgCl<sub>2</sub>, 100 mM Hepes (pH 7.9), 200  $\mu$ M sodium ascorbate, 600  $\mu$ M phenylmethylsulfonyl fluoride (PMSF), centrifuged again at 15,000g, and resuspended in reaction buffer (Fig. 1). Lane 2, Sup treated with IFN- $\alpha$  ( $2 \times 10^4$  units, 30 min at 30°C); lane 3, mixture of Mb and Sup treated with IFN- $\alpha$  (30 min at 30°C); lane 4, Mb treated with IFN- $\alpha$  (30 min at 30°C); lane 5, Mb treated with IFN- $\alpha$  (30 min at 30°C), then added to Sup on ice and incubated (30 min at 4°C); and lane 6, Mb incubated (30 min at 30°C) without IFN- $\alpha$  (CTL), added to Sup on ice, and incubated with IFN- $\alpha$  (30 min at 4°C). **(B)** Time course for IFN- $\alpha$ -induced ISGF3 formation with fractionated HeLa cell membranes. A membrane fraction from HeLa cell homogenates was prepared as in Fig. 2A. The membranes were incubated for the indicated times with IFN- $\alpha$  (lanes 1 to 7) or IFN- $\gamma$  (lane 8), placed on ice, and then mixed with the Sup fraction (30 min at 5°C). **(C)** IFN- $\alpha$ -induced formation of ISGF3 in a membrane fraction prepared from cells not treated with IFN- $\gamma$ . Homogenates of HeLa cells incubated with IFN- $\gamma$  or without IFN- $\gamma$  were prepared and incubated with IFN- $\alpha$  as in Fig. 1. Lane 1, homogenate prepared from cells exposed to IFN- $\gamma$  (12 hours) and incubated with IFN- $\alpha$  (30 min at 30°C); lane 2, same as lane 1 except the cells were not treated with IFN- $\gamma$ ; lane 3, the lysate assayed in lane 1 was centrifuged at 15,000g and the supernatant was incubated with IFN- $\alpha$  (30 min at 30°C); and lane 4, the lysate assayed in lane 2 was centrifuged at 15,000g and the sedimented material (Sed) was resuspended in the supernatant used in lane 3 and incubated with IFN- $\alpha$  (30 min at 30°C).

**Fig. 3.** Activation of a factor in a plasma membrane fraction by IFN- $\alpha$  that is *N*-ethylmaleimide (NEM) resistant and requires detergent to form ISGF3. **(A)** Activation of an NEM-resistant factor in the plasma membrane fraction that leads to the formation of ISGF3. Cell homogenates were prepared, fractionated, and assayed for formation of ISGF3 as in Fig. 2A. The indicated fractions were incubated with IFN- $\alpha$  (30 min at 30°C) (lanes 1 to 7). Lane 1, Sup; lane 2, Mb; lane 3, Mb added to the Sup (30 min at 5°C); lane 4, Mb treated with NEM; lane 5, Mb treated with NEM and mixed with the Sup (30 min at 5°C); lane 6, same as lane 5 except that the Sup had also been treated with NEM; lane 7, same as lane 3 except that the Sup was treated with NEM before it was added to the membranes. Fractions were treated with 10 mM NEM for 15 min at 20°C, and the reaction was stopped by the addition of 20 mM DTT (6). **(B)** Association of the IFN- $\alpha$ -activated factor with membranes. Membranes were incubated with IFN- $\alpha$  for 30 min and the suspension was centrifuged at 15,000g for 5 min. The supernatant of this sample was added to the Sup fraction on ice (lane 1). The sedimented membranes were resuspended in reaction buffer that contained NP-40 (0.1%) and centrifuged at 15,000g, and the supernatant from this sample was added to the Sup on ice (lane 2).

resuspended in reaction buffer. Membranes were incubated with or without IFN- $\alpha$  for 30 min. Addition of IFN- $\alpha$  to either the complete HeLa homogenate or to a combination of the Mb and Sup fractions induced the formation of ISGF3 (Fig. 2A). Neither the Mb nor the Sup fraction alone treated with IFN- $\alpha$  formed ISGF3. However, if the Mb fraction was incubated at 30°C with IFN- $\alpha$  before being mixed with the supernatant, which was maintained on ice, ISGF3 was formed. As a control, when membranes were initially incubated without IFN- $\alpha$  at 30°C and the membranes were subsequently exposed to both IFN- $\alpha$  and the Sup at 4°C, no ISGF3 was formed (Fig. 2A). These results reinforced the finding that activation of ISGF3 by IFN- $\alpha$  occurred in a cell-free system and suggested that (i) activation of the ISGF3 $\alpha$  proteins is a membrane-associated phenomenon and (ii) the Sup is needed only as a source of ISGF3 $\gamma$  because the amount of ISGF3 formed at 4°C did not change as a function of the time of incubation of the Mb and Sup mixture at 4°C (8). The kinetics of activation of ISGF3 in the Mb fraction were assessed to determine whether the rates of activation *in vitro* were similar to those *in vivo* (Fig. 2B). The Mb fraction was incubated 0 to 45 min in the presence of IFN- $\alpha$ , placed on ice, and then added to the Sup on ice. The formation of ISGF3 was detected within 2 min after IFN- $\alpha$  treatment, a time course similar to that observed *in vivo* (4, 6). Activation of ISGF3 $\alpha$  was also detected in membrane fractions prepared from HeLa cells not treated with IFN- $\gamma$  when such membranes were incubated with a supernatant fraction prepared from cells that had been exposed to IFN- $\gamma$  (Fig. 2C).

Because it appeared that activation of ISGF3 by IFN- $\alpha$  required a fraction containing primarily membranes, IFN- $\alpha$  probably interacted with its cell surface receptor and, through an unknown coupling mechanism, activated the ISGF3 $\alpha$  proteins. To further characterize the components in the membranes that participate in the activation of ISGF3 $\alpha$ , we took advantage of the fact that activated ISGF3 $\alpha$  is *N*-ethylmaleimide (NEM)-resistant, whereas ISGF3 $\gamma$  is NEM-sensitive (6). Membranes were incubated with IFN- $\alpha$  for 30 min, treated with or without NEM, and added to a supernatant fraction; the mixture was then assayed by EMSA (Fig. 3A). Because ISGF3 $\alpha$  is NEM-resistant, ISGF3 was also formed when the membranes were treated with NEM. If, however, the Sup was treated with NEM before the addition of IFN- $\alpha$ -treated membranes, no ISGF3 was detected. This is consistent with the observed NEM sensitivity of ISGF3 $\gamma$ . These data in conjunction with the observation that the ISGF3 complex can be formed at 4°C are



most consistent with the hypothesis that activation of the ISGF3 $\alpha$  proteins occurred in a cellular fraction that contains plasma membranes.

In vivo activation of ISGF3 $\alpha$  is associated with its translocation to the nucleus (4, 6). We therefore determined whether or not activation of ISGF3 $\alpha$  was directly coupled to its release from the membrane. After incubation with IFN- $\alpha$  for 30 min, the Mb fraction was sedimented at 15,000g for 15 min, and the supernatant from the membranes was added to the Sup fraction at 4°C (Fig. 3B). Under these conditions no ISGF3 was present. However, when the sedimented membranes were resuspended in buffer with the detergent NP-40, recentrifuged, and the supernatant then assayed, ISGF3 was formed. It can be inferred from this observation that release of the activating factor from the membranes is not directly coupled to the activation of the ISGF3 $\alpha$  proteins; rather, it appears to require another reaction.

A variety of cofactors that are needed for well-described signaling systems were included in the incubation buffer of the cell-free system (Fig. 1). Because only the Mb fraction was necessary to activate ISGF3 $\alpha$  in vitro, this provided an assay system to determine whether these cofactors were essential for the signaling process. Calcium had no effect on the activation of ISGF3. The removal of NaF, which inhibits serine-threonine phosphatases, was also without effect. Because the nonhydrolyzable guanosine triphosphate (GTP) analogue guanosine 5'-O-(3'-thiotriphosphate) (GTP- $\gamma$ -S) was not required and the presence of guanosine 5'-O-(2-thiodiphosphate) (GDP- $\beta$ -S, an inhibitor of GTP binding protein-mediated signaling) was without effect on activation of ISGF3 $\alpha$  by IFN- $\alpha$ , guanine nucleotide binding proteins do not appear to be required. The absence of ATP or the substitution of a nonhydrolyzable analogue adenylyl ( $\beta$ , $\gamma$ -methylene)-diphosphonate (AMP-PCP) for ATP inhibited activation of ISGF3 $\alpha$ , as did the addition of staurosporine (50 nM), a protein kinase inhibitor. Lower concentrations of staurosporine, which specifically inhibit protein kinase C's, had no effect on formation of ISGF3 (9). These results confirm the results of several in vivo studies that indicated that Ca<sup>2+</sup> is not needed for IFN- $\alpha$  signal transduction but that the activation of a protein kinase is required (10–13). However, the use of the in vitro system eliminates any secondary changes in cellular metabolism that these inhibitors might have caused in vivo.

Although much progress has been made in understanding the regulation of certain hormone-stimulated signaling systems, those systems that are activated by growth

factors and cytokines have been more difficult to analyze, in part because it has not been possible to duplicate any rapid response in vitro. IFN- $\alpha$ -induced ISGF3 formation is a very rapid event mediated by interaction of this cytokine with its cell surface receptor (1). We observed formation of ISGF3 with IFN- $\alpha$ -treated membranes mixed with the Sup fraction at 4°C; maximal formation of the complex required less than 30 min. Furthermore, the addition of staurosporine (50 nM) to membranes after incubation with IFN- $\alpha$  (30 min, 30°C) but before mixing with the Sup did not inhibit the formation of ISGF3 (8). The most likely explanation of our results is that the ISGF3 $\alpha$  proteins are membrane-associated and are rapidly activated in response to IFN- $\alpha$ . The activation of the factor in the membrane fraction appears not to be directly coupled to its release from the membrane fraction because only after the addition of detergent was a factor released that led to the formation of ISGF3. This suggests that another enzymatic reaction (for example the action of a protease or a lipase) may be needed to permit the association of ISGF3 $\alpha$  with ISGF3 $\gamma$ .

## IP<sub>3</sub> Receptor: Localization to Plasma Membrane of T Cells and Cocapping with the T Cell Receptor

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Immune responses in lymphocytes require cellular accumulation of large amounts of calcium (Ca<sup>2+</sup>) from extracellular sources. In the T cell tumor line Jurkat, receptors for the Ca<sup>2+</sup>-releasing messenger inositol 1,4,5-trisphosphate (IP<sub>3</sub>) were localized to the plasma membrane (PM). Capping of the T cell receptor-CD3 complex, which is associated with signal transduction, was accompanied by capping of IP<sub>3</sub> receptors. The IP<sub>3</sub> receptor on T cells appears to be responsible for the entry of Ca<sup>2+</sup> that initiates proliferative responses.

Signal transduction in many cellular systems, initiated by neurotransmitters, hormones, or antigens, involves an initial rapid rise in the concentration of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) followed by a slower plateau phase; the initial peak, but not the second phase, is independent of extracellular Ca<sup>2+</sup> (1). The phosphoinositide (PI) second messenger system is responsible for the initial release of intracellular Ca<sup>2+</sup> by the generation of IP<sub>3</sub>, which releases Ca<sup>2+</sup> from specific receptor proteins at intracellular sites

that are presumably associated with the endoplasmic reticulum (ER) (2).

In lymphocytes, the phase of elevated [Ca<sup>2+</sup>]<sub>i</sub> derived from external sources is more prolonged than in most other types of cells and leads to proliferation (3). The mechanisms responsible for this receptor-mediated influx of extracellular Ca<sup>2+</sup> have not been clarified. Inositol 1,3,4,5-tetrakisphosphate (IP<sub>4</sub>) has been suggested as a mediator (4). Alternatively, IP<sub>3</sub> may mediate this Ca<sup>2+</sup> entry either by communication of the IP<sub>3</sub>-responsive ER vesicles with the plasma membrane (PM) or by direct actions of IP<sub>3</sub> at receptors located on the PM (1, 5).

In Purkinje cells of the cerebellum, IP<sub>3</sub> receptors (IP<sub>3</sub>R) are concentrated in discrete components of the ER; there is no evidence for the receptor on the PM, as

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