Role of Protein Phosphorylation in Activation of Interferon-stimulated Gene Factors*

Sudip K. Bandyopadhyay and Ganes C. Sen‡

From the Department of Molecular Biology, The Cleveland Clinic Foundation, Cleveland, Ohio 44195-5285

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The possible involvement of protein phosphorylation in interferon (IFN)–mediated activation of IFN-stimulated gene factor 3 (ISGF3) was investigated. For this purpose, in vivo experiments with specific inhibitors of protein kinases and in vitro experiments with protein phosphatases were carried out. In HeLaM cells, 2-aminopurine, an inhibitor of double-stranded RNA-dependent protein kinase, blocked the induction of ISGF3γ subunit but not the activation of ISGF3α subunit. A series of experiments using combinations of protein and RNA-synthesis inhibitors and 2-aminopurine indicated that the block elicited by 2-aminopurine was at the level of ISGF3γ mRNA synthesis. Activation of ISGF3α, although insensitive to 2-aminopurine, was completely blocked by 10 µM staurosporine, an inhibitor of protein kinase C. On the other hand, even 500 nM staurosporine did not block the induction of ISGF3γ.

Incubation of cytoplasmic or nuclear extracts of IFN-treated HeLaM cells in vitro with alkaline phosphatase completely eliminated their ability to form the ISGF3 complex but not the ISGF1 complex. Treatment with acid phosphatase, on the other hand, changed the electrophoretic mobility of the ISGF3 complex but did not obliterate it. Complementation experiments revealed that ISGF3α was the alkaline phosphatase-sensitive component of the complex. These results suggest that a protein kinase C–mediated phosphorylation step is involved in ISGF3α activation and an α-2-aminopurine-sensitive component is involved in ISGF3γ mRNA induction.

The biological actions of interferons (IFNs) are mediated by the proteins encoded by the IFN-responsive genes (1–3). IFN-α and IFN-β presumably bind to the same cell surface receptors and induce the expression of a variety of genes (4). IFN-γ binds to a different receptor and induces another set of genes which partially overlap with those induced by IFN-α/β. The process of gene induction by IFN-α/β operates primarily at the transcriptional level (5). Recent studies have identified a cis-acting sequence, the interferon-stimulated response element (ISRE), present in the 5′-flanking region of all IFN-α/β-inducible genes examined so far (6–15). In vitro transfection experiments with chimeric genes have established that the presence of ISRE is sufficient to mediate the transcriptional induction signal elicited by IFN-α/β (10, 16–22).

At least three trans-acting factors, present in the nuclei of IFN-α-treated cells, bind to ISRE (6, 10, 11, 13, 23, 24). Electrophoretic mobility shift assays and DNA footprinting analyses have been used to study these IFN-stimulated gene factors (ISGF). The fastest moving complex, termed ISGF1, is present in both IFN-α-treated and untreated cells. ISGF2 is induced by both IFN-α and IFN-γ, and its appearance requires ongoing protein synthesis during IFN treatment (10, 23). Recently, the cDNA for ISGF2 has been cloned and sequenced, thereby revealing its identity with IRF-1 (25), a trans-acting factor involved in the transcription of the β-IFN gene (26). The third factor, ISGF3, is induced by IFN-α but not by IFN-γ, and it appears to be the crucial positive regulatory factor which is responsible for transcriptional activation of the IFN-α-responsive genes (10).

Activation of ISGF3 occurs in the cytoplasm of cells within minutes of contact with IFN-α (27, 28). In untreated cells, active ISGF3 cannot be detected either in the cytoplasmic or nuclear fractions. Upon IFN-α treatment, active ISGF3 appears first in the cytoplasm and then it translocates to the nucleus where it presumably binds to the ISREs of different IFN-inducible genes and thereby activates their transcription.

Thus, ISGF3 itself physically transduces the signal from the cytoplasm to the gene. Understanding the mechanism of activation of ISGF3 in the cytoplasm of IFN-α-treated cells is therefore crucial for defining the signal transduction pathway of IFN-α.

ISGF3 is composed of two functional subunits, ISGF3γ and ISGF3α, both of which are needed for efficient DNA binding (28). In most cells, there is a high constitutive level of ISGF3γ, but HeLaM cells do not contain a detectable level of ISGF3γ (21). We have used this cell line to delineate some details of ISGF3 activation by IFN-α. ISGF3α, which is present in an inactive form in the cytoplasm, can be activated within minutes of cellular contact with IFN-α. This activation does not need new protein synthesis (21). As a result, in cells having a high constitutive level of ISGF3γ, the overall process of ISGF3 activation and the resultant induction of gene transcription do not need ongoing protein synthesis. This is not the case, however, for HeLaM cells. In these cells, ISGF3 activation requires, in addition to ISGF3α activation, induced synthesis of ISGF3γ subunit (21). We and others (21, 29) have shown that ISGF3γ synthesis can be induced by either IFN-α or IFN-γ. IFN-γ, however, cannot activate ISGF3α.

An in vitro complementation assay has been developed for measuring the levels of active ISGF3α and active ISGF3γ in
cell extracts (21). We have operationally defined two distinct signals elicited by IFN-α. Signal 1, which is also produced by IFN-γ, induces the synthesis of ISGF3γ, whereas signal 2 activates the inactive precursor of ISGF3α. The two putative signals have different sensitivities to several inhibitors such as cycloheximide and 2-amino purine (2AP). Using the complementation assay, we have shown that ISGF3γ is required for the nuclear translocation of activated ISGF3α (21). We have also shown activated ISGF3α in the absence of ISGF3γ, has a relatively short half-life in the cytoplasm. The activation characteristics of ISGF3α, namely its rapidity, its reversibility, and its independence of protein synthesis, strongly suggest that the activation-inactivation equilibrium is regulated by a specific IFN-α-induced post-translational modification of this protein (21). Protein phosphorylation could be such a specific modification.

There are several reports in the literature suggesting the involvement of protein phosphorylation in IFNs signal transduction (36-35). A possible role of protein kinase C in this process has been strongly implicated. Inhibitors of this enzyme have been shown to block signal induction by IFN-α and the antiviral response of IFN-α treated cells (31-35). Down-regulation of protein kinase C from the cell surface by prolonged treatment of the cells with phorbol esters (36) also blocks IFNs antiviral actions (37, 35). Recently, it has been reported that inhibitors of protein kinase C blocks the activation of ISGF3 by IFN-α (32). We have reported previously that in HeLaM cells, 2AP, an inhibitor of the double-stranded RNA-dependent protein kinase, specifically inhibits gene induction by both IFN-α and IFN-γ (38). It was shown that the production or the function of signal 1 elicited by either IFN was blocked by 2AP, whereas signal 2 production by IFN-α was not affected. As expected, ISGF3 activation in HeLaM cells was sensitive to 2AP. Complementation assays revealed that the induction of ISGF3γ by signal 1 was blocked by 2AP, whereas activation of ISGF3α by signal 2 was not affected (21).

In this paper, we provide further evidence for the involvement of protein phosphorylation in ISGF3 activation process. Our experiments demonstrated that 2AP specifically inhibited the synthesis of ISGF3γ mRNA, thereby suggesting a role of protein phosphorylation in this process. We also showed that 10 nm staurosporine, an inhibitor of protein kinase C, blocked the activation of ISGF3α. The same inhibitor, even at 500 nM concentration, did not block the induction of ISGF3γ by either IFN-α or IFN-γ. Treatment of ISGF3 with alkaline phosphatase, in vitro, abolished its DNA-binding ability. The component affected by this treatment was ISGF3α but not ISGF3γ.

**RESULTS**

2-Aminopurine Blocks ISGF3γ mRNA Synthesis—We have previously shown that ISGF3γ induction by either IFN-α or IFN-γ needs ongoing protein synthesis and it is blocked by 2AP (21). In a series of new experiments we have analyzed this induction process further in order to be able to identify the 2AP-sensitive step in this process. A hypothetical working model for the induction of active ISGF3γ is shown in Fig. 1. According to this model IFN-γ binds to its receptor and elicits a signal in step 1 which persists even after IFN is removed. This signal travels to the nucleus and induces transcription of the ISGF3γ gene to produce ISGF3γ mRNA in step 2. In step 3, the mRNA is translated into ISGF3γ protein which may be activated by a post-translation modification in step 4. We wanted to test which of these steps is sensitive to inhibition by 2AP. For this purpose, assays were devised to study these steps individually, using inhibitors of protein and mRNA syntheses. A previously described complementation

![Fig. 1. Possible sequential steps in the process of ISGF3γ induction by IFNγ in HeLaM cells. IFNγ binds to a specific receptor on the cell surface and transmits a signal to the ISGF3γ gene in the nucleus. As a result, in step 2, ISGF3γ mRNA is transcribed from the ISGF3γ gene. In step 3, ISGF3γ mRNA is translated to the ISGF3γ protein. In step 4, functional ISGF3γ* is formed by a post-translational modification.](https://example.com/Fig1.png)
Protein Phosphorylation in IFN Signal Transduction

assay was used for measuring functional ISGF3γ (21). ISGF3α was supplied from a cytoplasmic extract of HeLaM cells treated with IFN-α and CHX. As shown in Fig. 2A, an IFN-γ-treated cell extract when supplemented with ISGF3α can form the ISGF3 complex (lane 2). ISGF3γ was not present in extracts of cells treated with IFN-γ + CHX (lane 3). On the other hand, when cells were first treated with IFN-γ + CHX, the additives were removed and the incubation was continued in untreated culture medium, active ISGF3γ was formed (lane 4). During the follow-up incubation new mRNA synthesis was not needed since the presence of actinomycin D did not form the ISGF3 complex with our working model (Fig. 1). and they show that different steps of the induction process can be studied separately. In the next experiment, effects of 2AP on different steps of the induction process were examined. The overall ISGF3γ induction process was inhibited by 2AP (Fig. 2B, lane 1). However, if cells were treated with IFN-γ and 2AP, the agents were removed by washing, and incubation was continued, ISGF3γ induction was not blocked (Fig. 2B, lane 5). This observation indicates that step 1 of our working model for ISGF3γ induction was not blocked by 2AP. When ISGF3γ mRNA synthesis was allowed to occur by treating the cells with IFN-γ and CHX, 2AP could not prevent the subsequent formation of active ISGF3γ after the removal of IFN-γ and CHX (Fig. 2B, lane 3). This result indicates that 2AP does not have any effect on steps 3 and 4 of the induction process. By elimination, it appears that step 2, at which ISGF3 γ mRNA is synthesized in response to a signal elicited by IFN-γ, is the step affected by 2AP. Several observations supported this conclusion. When cells were treated with IFN-γ and 2AP followed by an incubation in the presence of CHX only, no active ISGF3γ was formed(Fig. 2B, lane 2), suggesting that the 2AP-mediated block is prior to step 3. As expected, inclusion of 2AP during the first phase of incubation in the presence of IFN-γ and CHX did not block ISGF3γ induction (Fig. 2B, lane 7). These results are consistent with 2AP blocking step 2 of the working model (Fig. 1). 2AP does not block step 1 in which a IFN-γ-elicited signal is produced which lasts after IFN-γ is removed. 2AP also does not block the translation of ISGF3γ protein or its putative activation by post-translational modification. The step sensitive to 2AP is step 2, at which ISGF3γ mRNA is synthesized. In accord with this conclusion we observed that no active ISGF3γ was formed if mRNA synthesis was blocked by actinomycin D during the second phase of incubation following a first phase of incubation with IFN-γ and 2AP (data not shown), again suggesting that no ISGF3γ mRNA had been synthesized in the presence of 2AP.

Stauroporine Blocks ISGF3α Activation—Stauroporine is a specific inhibitor of protein kinase C. At a relatively high concentration, it has been shown to inhibit IFN-inducible gene transcription (32). This inhibition has been traced to a lack of activation of ISGF3 in cells treated with IFN-α in the presence of stauroporine. In the experiments shown in Fig. 3, we examined if ISGF3α activation or ISGF3γ induction or both were sensitive to stauroporine in HeLaM cells. ISGF3α activation was blocked by stauroporine at 10 and 50 nM concentrations (Fig. 3A, lanes 4 and 5) or even at 500 nM concentration (data not shown). When cells were treated with IFN-α and CHX for 30 min followed by a treatment with stauroporine (500 nM) for 30 min, active ISGF3α was not present in the extract of active ISGF3α is short and its reactivation is blocked by stauroporine. Induction of ISGF3γ followed by a treatment with stauroporine, on the other hand, did not obliterate its activity (Fig. 3A, lane 9).

ISGF3γ can be induced not only by IFN-γ but also by IFN-α. We investigated if its induction by IFN-α was also insensitive to stauroporine. As expected, IFN-α induced ISGF3 and either CHX or stauroporine blocked this induction process (Fig. 3B, lanes 3-5). However, when assayed for ISGF3γ induction or ISGF3α activation individually, it was apparent that ISGF3γ was induced by IFN-α in the presence of 500 nM stauroporine (Fig. 3B, lane 2), whereas ISGF3α was activated in the presence of CHX. These results demonstrated that although a very low dose of stauroporine (10 nM) can block the activation of ISGF3α, even a relatively high dose (500 nM) of it cannot block the induction of ISGF3γ by either IFN-α or IFN-γ.

PMA Does Not Affect ISGF3α Activation—Since ISGF3α activation was blocked by stauroporine, a known inhibitor of protein kinase C, we wondered whether PMA, which activates protein kinase C and eventually down-regulates it from the cell surface, will affect ISGF3α activation. HeLaM cells were treated with PMA for 1 h to activate protein kinase C and then treated with IFN-α and CHX for ISGF3α activation. The short treatment with PMA did not block ISGF3α activation (Fig. 4, lane 3). It also did not block ISGF3γ induction by IFN-α, since treatment with PMA followed by IFN-α resulted in active ISGF3 formation (Fig. 4, lane 4). PMA treatment by itself, however, did not activate ISGF3α (Fig. 4, lane 5). When cells were treated for 24 h with PMA to completely down-regulate protein kinase C from the cell surface, activation of ISGF3α (Fig. 4, lane 7) and induction of
Fig. 3. Effect of staurosporine on ISGF3 activation and ISGF3 induction. For complementation assays, ISGF3γ was provided by extracts of cells treated with IFNγ (γ) for 16 h, and ISGF3α was provided by extracts of cells treated with IFNα and CHX (CHX + α) for 30 min. Additional treatments of cells and the compositions of mixtures in each lane are indicated on the figure. A: lane 8, mixture of an extract containing ISGF3γ and a cytosolic extract of cells treated with IFNα + CHX for 30 min followed by a treatment with 500 nM staurosporine for 30 min. Lane 9, mixture of an extract of IFNα + CHX-treated cells and an extract of cells treated with IFNγ for 16 h followed by a treatment with 500 nM staurosporine for 30 min. B: lane 2, mixture of an extract of cells treated with IFNα + 50 nM staurosporine for 12 h and an extract of cells treated with IFNα + CHX for 30 min.

ISGF3γ (Fig. 4, lane 8) were not affected.

Fig. 4. Effects of PMA on ISGF3α activation. ISGF3γ was provided by IFNγ-treated cell extracts, where indicated. Lanes 2 and 3, cells were treated with PMA (81 nM) for 1 h followed by a treatment with IFNα + CHX for 30 min; lane 4, cells were treated with 81 nM PMA for 1 h and then with IFNα for 3 h; lane 5, cells were treated with PMA for 4 h; lane 7, cells were treated with 324 nM PMA for 24 h and then with IFNα + CHX for 30 min; lane 8, cells were treated with 324 nM PMA for 24 h and then with IFNα for 3 h.

Effects of in Vitro Dephosphorylation of ISGF3—Our observations that inhibitors of different protein kinases block the activation of ISGF3 in vivo suggested that this process involves phosphorylation of ISGF3. Consequently, we argued that dephosphorylation of activated ISGF3 should affect its ISRE binding activity. We tested this possibility by incubating in vitro ISGF3-containing nuclear extracts with acid phosphatase or alkaline phosphatase before examining their ISRE binding activities (Fig. 5). Acid phosphatase treatment enhanced the mobility of the ISGF3 complex as well as that of part of the ISGF1 complex (Fig. 5, lane 2). Alkaline phosphatase treatment, on the other hand, did not affect the ISGF1 complex, but formation of the ISGF3 complex was totally blocked in such an extract (Fig. 5, lane 3).

Next, we examined if the cytoplasmic form of ISGF3 was also sensitive to the action of phosphatases and if the sensitivity remained after the formation of the protein-DNA complex. In one experiment, cytoplasmic extracts of IFN-treated cells were treated with increasing concentrations of acid phosphatase or alkaline phosphatase before examining their ISRE binding activity. We tested this possibility by incubating in vitro ISGF3-containing nuclear extracts with acid phosphatase or alkaline phosphatase and then with IFNα for 30 min. The extract was either untreated (lane 1) or treated with 10.0 μg/ml of acid phosphatase (lane 2) or 12.5 μg/ml of alkaline phosphatase (lane 3) for 20 min at 22°C before the addition of the probe. Acid phosphatase was dissolved in 50 mM sodium acetate, pH 5.2, and alkaline phosphatase was either added directly or diluted with 20 mM Tris hydrochloride, pH 8.0, immediately before use. Since phosphatases would have removed the labeled terminal phosphate from a kinase-labeled probe, for this experiment the probe was labeled with [32P]dATP by filling in using Klenow polymerase.

In another experiment, ISGF3-containing cytoplasmic extracts of cells treated with increasing concentrations of acid phosphatase were incubated with 50 nM staurosporine for 24 h and then with IFNα for 30 min. Fig. 5. Effects of phosphatase treatments of nuclear extracts on ISGF3 complex formation. Nuclear extracts were prepared from cells treated with IFNγ for 16 h followed by IFNα for 30 min. The extract was either untreated (lane 1) or treated with 10.0 μg/ml of acid phosphatase (lane 2) or 12.5 μg/ml of alkaline phosphatase (lane 3) for 20 min at 22°C before the addition of the probe. Acid phosphatase was dissolved in 50 mM sodium acetate, pH 5.2, and alkaline phosphatase was either added directly or diluted with 20 mM Tris hydrochloride, pH 8.0, immediately before use. Since phosphatases would have removed the labeled terminal phosphate from a kinase-labeled probe, for this experiment the probe was labeled with [32P]dATP by filling in using Klenow polymerase.

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ISGF3 and then challenged with the labeled DNA probe for complex formation (Fig. 6, lanes 2–4). As was observed with the nuclear extract, acid phosphatase treatment caused a clear shift in the mobility of the ISGF3 complex. The new complex moved slightly faster than ISGF3. Increasing the phosphatase concentration increased the proportion of the faster form, which appeared as a distinct tight band. The acid phosphatase treatment, even at the highest concentration, did not perturb the formation or the mobility of the constitutive complex which had a much faster mobility. Exactly similar results were obtained when the extracts were first incubated with the labeled probe to allow complex formation and then treated with acid phosphatase at increasing concentrations (Fig. 6, lanes 5–7). These results suggest that acid phosphatase treatment removes specific and probably the same phosphate residues from the ISGF3 complex, irrespective of whether it is bound to DNA or not, and the removal of these residues does not affect the DNA binding property of the complex, although it changes its electrophoretic mobility. In another experiment, we tested the sensitivity of cytoplasmic extracts to alkaline phosphatase treatment before and after incubating with the labeled DNA probe (Fig. 6, lanes 8–11). This treatment obliterated the ISGF3 complex in a dose-dependent manner, both before and after DNA binding, suggesting that it removed specific phosphate residues from the proteins in ISGF3 which are essential for binding of this complex to DNA. It is curious to note that the formation of the faster constitutive complex was enhanced by alkaline phosphatase treatment.

**ISGF3α Is the Alkaline Phosphatase-sensitive Component of ISGF3**—Once we established that the DNA-binding activity of ISGF3 could be eliminated by alkaline phosphatase treatment, we tested if the ISGF3α component or the ISGF3γ component or both were affected by this treatment. For this purpose, ISGF3γ-containing extract of IFN-γ-treated HeLaM cells and ISGF3α-containing extract of IFN-α and CHX-treated HeLaM cells were separately treated with alkaline phosphatase and tested for activities using the complementation assay (Fig. 7). To carry out this experiment successfully, it was necessary to completely remove alkaline phosphatase from the treated extract before the complementation assay was performed. This was achieved by using alkaline phosphatase bound to an insoluble matrix as described in detail under “Experimental Procedures.” When neither component was phosphatase-treated, ISGF3 complex

**Fig. 6. Effects of phosphatase treatments of cytoplasmic extracts before and after DNA binding.** A cytoplasmic extract of cells treated as in Fig. 5 was used for this experiment. It was incubated with various amounts of acid and alkaline phosphatases before or after an incubation with labeled DNA probe. The concentrations of phosphatases were 2.5 μg/ml (lanes 2 and 5), 5.0 μg/ml (lanes 3 and 6), 10.0 μg/ml (lanes 4 and 7), 5.0 μg/ml (lanes 8 and 10), and 12.5 μg/ml (lanes 9 and 11). Lane 1 was not treated with any phosphatase. Other conditions were the same as in Fig. 5.

**Fig. 7. Effects of phosphatase treatment of ISGF3α and ISGF3γ components individually.** The compositions of extracts used in each lane are indicated on the figure. Lane 1, ISGF3γ mixed with ISGF3α; lane 2, alkaline phosphatase-treated ISGF3γ mixed with untreated ISGF3α; lane 3, same as in lane 2 but supplemented with untreated ISGF3γ after phosphatase removal; lane 4, alkaline phosphatase-treated ISGF3α mixed with untreated ISGF3γ; lane 5, same as in lane 4 but supplemented with untreated ISGF3α after phosphatase removal.

was formed, as expected, upon complementation (Fig. 7, lane 1). Treatment of the ISGF3γ component with alkaline phosphatase did not affect its ability to form the ISGF3 complex (Fig. 7, lane 2). Supplementation with additional untreated extract containing ISGF3γ did not have any major effect (Fig. 7, lane 3). Treatment of the ISGF3α-containing extract with alkaline phosphatase, on the other hand, completely abolished the formation of the ISGF3 complex (Fig. 7, lane 4), suggesting that ISGF3α was the phosphatase-sensitive component. To establish that complete removal of phosphatase had been achieved by our procedures, the same extract was supplemented with a source of active untreated ISGF3α, in addition to the usual addition of ISGF3γ, and tested for ISGF3 complex formation (Fig. 7, lane 5). The usual amount of ISGF3
complex was formed thereby confirming the validity of our assay system. It is also worth noting that unbound labeled DNA probe was present in all lanes. If alkaline phosphatase had not been removed completely, it would have removed the terminal labeled phosphate of the DNA probe, and the released labeled phosphate would have migrated out of the gel under the conditions of electrophoresis used here. As mentioned earlier, formation of the faster migrating constitutive complex was not affected by the phosphatase treatment.

DISCUSSION

In our early studies with HeLaM cells, we observed that gene induction by IFN-α required ongoing protein synthesis which could be obviated by pretreating the cells with IFN-γ (39, 41). According to our working model (21, 38, 39), both IFNs produce a hypothetical signal, signal 1, which induces the transcription of a gene, X. The product, mRNA, needs to be translated to the corresponding protein, protein X for functioning of signal 1. As would be expected from this model, inhibition of either mRNA synthesis or protein synthesis blocked this pathway. Our recent results strongly indicate that protein X is identical to ISGF3γ (21). We have shown that induction of protein X or ISGF3γ by either IFN-α or IFN-γ is blocked by 2AP. In the experiments reported here, we further dissected this process and tried to identify the exact step in the induction pathway of ISGF3γ which is sensitive to 2AP. We operationally divided this induction process into four discrete steps which can be assayed individually. Our results clearly indicated that the 2AP-mediated block is at the level of ISGF3γ mRNA synthesis. There are obviously multiple steps within what has been called step 2 here. Such steps may involve activation of specific trans-acting factors, their binding to cognate cis-acting elements of ISGF3γ gene, and their interactions with other proteins involved in the transcription process. Testing of each of these steps for 2AP sensitivity awaits the development of reagents such as ISGF3γ cDNA and genomic clones. Nonetheless, the experiments reported here established that the action of a specific protein kinase is required at one or more steps of this process. Whether the kinase in question is the double-stranded RNA-dependent protein kinase remains to be seen.

Several reports in the literature implicated the involvement of protein kinase C in IFN-α's signal transduction (30–35). These studies mostly used various inhibitors of this enzyme and data not shown). Another major distinction of the ISGF3γ gene as an IFN-α-inducible gene was unraveled by experiments reported in this paper. Staurosporine inhibited ISGF3α activation by IFN-α. As a result, transcriptional induction of IFN-α-inducible genes was blocked by this inhibitor. Induction of ISGF3γ by IFN-α was, on the other hand, totally insensitive to staurosporine. It appears therefore that, at least in HeLaM cells, transcriptional induction of the ISGF3γ gene by IFN-α is mediated by an alternative route which requires neither the ISGF3γ protein nor active ISGF3α. In principle, there could be other as yet untested IFN-α-inducible genes which share the induction pathway of ISGF3γ gene. One can speculate that these genes are activated not by ISGF3 but by some other trans-acting factor which binds to their ISREs. It is also conceivable that their induction is mediated not by ISRE but by a different cis-acting element. All genes carrying this putative cis-element may be directly inducible by both IFN-α and IFN-γ without the involvement of protein kinase C.

Drawing upon the experimental observations noted in this paper and elsewhere (3), one can draw the following model for gene induction by IFN-α in HeLaM cells (Fig. 8). IFN-α binds to its cell surface receptor and initiates a chain reaction which involves a specific isoform of protein kinase C. This leads to the phosphorylation of ISGF3α, in the cytoplasm, which activates this protein and results in its binding to ISGF3γ and translocation to the nucleus. Activated ISGF3α can be readily inactivated by dephosphorylation, and hence it has a short functional half-life. It is not known whether the phosphorylation state of ISGF3α affects its binding to
ISGF3γ or its functioning in DNA binding. A different signal generated by IFN-α transmits to the nucleus by an unknown mechanism and induces the transcription of ISGF3γ mRNA. Functioning of this signal, but not its generation, is sensitive to the protein kinase inhibitor ZAP. This suggests that a different protein kinase, not protein kinase C, is involved in the alternative pathway of IFN-α-elicited signal transduction. IFN-γ, which binds to a different receptor, can also generate a ISGF3γ mRNA-inducing signal which is indistinguishable from the corresponding signal generated by IFN-α. This alternative pathway of IFN-α-elicited signal transduction is sensitive, but not its generation, is sensitive to the protein kinase inhibitor ZAP. This suggests that a different protein kinase, not protein kinase C, is involved in the alternative pathway of IFN-α-elicited signal transduction. IFN-γ, which binds to a different receptor, can also generate a ISGF3γ mRNA-inducing signal which is indistinguishable from the corresponding signal generated by IFN-α.

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REFERENCES
1. Lengyel, P. (1982) Annu. Rev. Biochem. 51, 251-282
2. Pestka, S., Langer, J. A., Zoon, K. C., and Samuel, C. E. (1987) Annu. Rev. Biochem. 56, 727-777
3. Sen, G. C. (1991) in The Hormonal Control of Gene Transcription (Cohen, P., and Foulkes, J. G., eds) pp. 349-374, Elsevier Science Publishers B. V., Amsterdam
4. Revel, M., and Chebath, J. (1986) Trends Biochem. Sci. 11, 166-170
5. Friedman, R. L., Manly, S. P., McMahon, M., Kerr, I. M., and Stark, G. R. (1984) EMBO J. 3, 745-755
6. Cohen, B., Peretz, D., Vaiman, D., Benech, P., and Chebath, J. (1988) EMBO J. 7, 1411-1419
7. Hug, H., Costas, M., Stashel, P., Aebi, M., and Weissmann, C. (1988) Mol. Cell. Biol. 8, 3065-3079
8. Israel, A., Kimura, A., Fournier, A., Fellous, M., and Kourilsky, P. (1986) Nature 322, 743-746
9. Korbet, B., Hood, L., and Stroynowski, I. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3380-3384
10. Levy, D. E., Kessler, D. S., Pine, R., Reich, N., and Darnell, J. E., Jr. (1988) Genes & Dev. 2, 383-393
11. Porter, A. C. G., Chernajovsky, Y., Dale, T. C., Gilbert, C. S., Stark, G. R., and Kerr, I. M. (1988) EMBO J. 7, 85-92
12. Reich, N., Evans, B., Levy, D., Knight, E. K., Blomstrom, D., and Darnell, J. E., Jr. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6394-6398
13. Rutherford, M. N., Hannigan, G. E., and Williams, B. R. G. (1988) EMBO J. 7, 751-759
14. Samanta, H., Engel, D. A., Chao, H. M., Thakur, A., Garcia-Blanco, M. A., and Lengyel, P. (1986) J. Biol. Chem. 261, 11849-11858
15. Wathiele, M. G., Claus, I. M., Nols, C. B., Content, J., and Hue, G. A. (1987) Eur. J. Biochem. 169, 313-321
16. Cohen, B., Vaiman, D., and Chebath, J. (1989) Nucleic Acids Res. 17, 1679-1696
17. Grishkoff, G., Toniato, E., Engel, D. A., and Lengyel, P. (1987) J. Biol. Chem. 262, 11878-11883
18. Reid, L. E., Brasnett, A. H., Gilbert, C. S., Porter, A. C. G., Gewert, D. R., Stark, G. R., and Kerr, I. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 840-844
19. Lew, D. J., Decker, T., and Darnell, J. E., Jr. (1989) Mol. Cell. Biol. 9, 5404-5411
20. Raj, N. B. K., Engelhardt, J., Au, W. C., Levy, D. E., and Pitha, P. M. (1989) J. Biol. Chem. 264, 16558-16666
21. Bandyopadhyay, S. K., Kalvakolanu, D. V. R., and Sen, G. C. (1990) Mol. Cell. Biol. 10, 5056-5063
22. Kalvakolanu, D. V. R., Bandyopadhyay, S. K., Tiwari, R. K., and Sen, G. C. (1991) J. Biol. Chem. 266, 873-879
23. Kessler, D. S., Levy, D. E., and Darnell, J. E., Jr. (1986) Proc. Natl. Acad. Sci. U. S. A. 85, 8521-8525
24. Dale, T. C., Rosen, J. M., Guille, M. J., Lewin, A. R., Porter, A. G. C., Kerr, I. M., and Stark, G. R. (1989) EMBO J. 8, 831-839
25. Pine, R., Decker, T., Kessler, D. S., Levy, D. E., and Darnell, J. E., Jr. (1990) Mol. Cell. Biol. 10, 2445-2457
26. Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T., and Taniguchi, T. (1988) Cell 54, 903-913
27. Dale, T. C., Imam, A. M. A., Kerr, I. M., and Stark, G. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1203-1207
28. Levy, D. E., Kessler, D. S., Pine, R., and Darnell, J. E., Jr. (1989) Genes & Dev. 3, 1362-1371
29. Levy, D. E., Lew, D. J., Decker, T., Kessler, D. S., and Darnell, J. E., Jr. (1990) EMBO J. 9, 1105-1111
30. Fan, X., Goldberg, M., and Hloon, B. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5122-5125
31. Pfeffer, L. M., Strulovich, B., and Saltiel, A. R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6537-6541
32. Reich, N. C., and Pfeffer, L. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8761-8765
33. Faltynek, C. R., Frincker, G. L., Gusella, G. L., Varesio, L., and Radzioch, L. (1989) J. Biol. Chem. 264, 14303-14311
34. Erusalimsy, J. D., Keffer, R. F., Gilmore, D. J., and Milstein, C. (1988) Proc. Natl. Acad. Sci. U. S. A. 86, 1973-1976
35. Akai, H., and Larner, A. C. (1989) J. Biol. Chem. 264, 3252-3255
36. Bandyopadhyay, S. K., and Bancroft, C. (1989) J. Biol. Chem. 264, 14216-14219
37. Young, S., Parker, P. J., Ullrich, A., and Stabel, S. (1987) Biochim. J. 244, 775-779
38. Tiwari, R. K., Kusari, J., Kumar, R., and Sen, G. C. (1988) Mol. Cell. Biol. 8, 4289-4294
39. Kusari, J., and Sen, G. C. (1986) Mol. Cell. Biol. 6, 2062-2067
40. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475-1489
41. Kusari, J., and Sen, G. C. (1987) Mol. Cell. Biol. 7, 528-531