Cytoplasmic activation of ISGF3, the positive regulator of interferon-α-stimulated transcription, reconstituted in vitro

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The signal transduction pathway through which interferon-α (IFNα) stimulates transcription of a defined set of genes involves activation of DNA-binding factors specific for the IFNα-stimulated response element (ISRE). IFN-stimulated gene factor-3 (ISGF3), the positive regulator of transcription, was derived in response to IFNα treatment from preexisting protein components that were activated first in the cell cytoplasm prior to appearance in the nucleus. Nuclear translocation of ISGF3 required several minutes and could be inhibited by NaF. Formation of active ISGF3 was mimicked in vitro by mixing cytoplasmic extracts from IFNα-stimulated cells with extracts of cells treated to contain high amounts of the unactivated factor. Active ISGF3 was found to be formed from association of two latent polypeptide precursors that were distinguished biochemically by differential sensitivity to *N*-ethyl maleimide. One precursor was modified in response to IFNα occupation of its cell-surface receptor, thus enabling association with the second subunit. The resulting complex then was competent for nuclear translocation and binding to ISRE. Cytoplasmically localized transcription factor precursors thus serve as second messengers to translate directly an extracellular signal into specific transcriptional activity in the nucleus.

[*Key Words*: Signal transduction; interferon-stimulated genes; transcription; DNA binding; cytoplasmic activation; in vitro reconstitution]

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Cellular responses to changes in the extracellular environment require information transfer from the exterior of the cell to the interior of the cell. When extracellular stimuli elicit transcriptional responses, the signal transduction process reports information from the cell exterior to the nucleus. Responses to small molecules such as ions, heavy metals, and steroids, or to physical agents such as heat may be mediated by direct transmission of the inducing agent into the cell, in some cases into the nucleus (Maniatis et al. 1987; Evans 1988; Furst et al. 1988; Minghetti and Norman 1988; Westin and Schaffner 1988). However, a profoundly different situation is presented by responses to polypeptide stimuli, and perhaps also by inductions dependent on cell–cell or cell–matrix contact, in which internalization of the ligand is not required for transcriptional stimulation. In a growing number of cases, rapid changes in expression of limited sets of genes have been linked to occupation of specific cell-surface receptors by polypeptide ligands (Friedman et al. 1984; Greenberg and Ziff 1984; Larner et al. 1984; Friedman and Stark 1985; Lau and Nathans 1985, 1987; Zullo et al. 1985; Greenberg et al. 1985; Prywes and Roeder 1986; Almendral et al. 1988) or to direct cell–cell contact (Clayton and Darnell 1983). Because many cytokines and growth factors lead to fluctuations in the levels of one or more small molecule second messengers in target cells, it is possible that signal transduction is mediated through the actions of specific protein kinases affected by these agents (Kikkawa and Nishizuka 1986; Nishizuka 1986; Rozengurt 1986; Berridge 1987; Yamamoto et al. 1988). Although it has been shown that agents that artificially perturb the intracellular levels of second messengers can lead to changes in gene transcription (Greenberg et al. 1986a,b; Fisch et al. 1987; Pine et al. 1988; Riabowol et al. 1988), it is not clear at all how the specificity of each individual ligand for stimulating a limited set of genes can be maintained if the signal passes through such broadly affected pathways. We have been studying the regulation of gene expression by interferon-α (IFNα) with the ultimate goal of understanding the specific molecules involved in one such signal response pathway.

Interferons-α and -β (IFNα IFNβ) were among the first polypeptide ligands demonstrated to regulate directly and rapidly transcription of a limited set of specific target genes in human cells (Friedman et al. 1984;

Larner et al. 1984, 1986; Friedman and Stark 1985; Levy et al. 1986). Although IFNα-stimulated genes (ISGs) are characterized as primary response genes (i.e., IFNα treatment triggers transcriptional induction that is detectable within 5–10 min and is mediated by preexisting protein components), it has not been possible to document any involvement of known second messengers, kinases, or previously identified transcription factors in this response (Larner et al. 1984; Tamm et al. 1987; D. Lew et al., unpubl.). Our previous studies have implicated an IFN-stimulated DNA-binding protein (ISGF3) as the positive transcriptional activator of ISGs (Levy et al. 1988a). This factor recognizes a conserved regulatory sequence, the IFNα-stimulated response element (ISRE), present in all ISGs characterized to date (Israel et al. 1986, 1987; Vogel et al. 1986; Gribaudo et al. 1987; Reich et al. 1987; Wathelet et al. 1987; Cohen et al. 1988; Levy et al. 1988a,b; Porter et al. 1988; Rutherford et al. 1988; Shirayoshi et al. 1988; Reid et al. 1989). The ISRE acts as an orientation-independent IFN-dependent enhancer element (Reich et al. 1987) and serves as the binding site in vitro and probably in vivo (Kessler et al. 1988a; Levy et al. 1988a; Dale et al. 1989b) for two IFNα-induced protein factors as well as for at least one constitutive factor.

ISGF3 has been implicated as the ISG transcriptional activator: its binding activity appears in cells immediately after IFNα treatment with no requirement for de novo protein synthesis (Levy et al. 1988a). Extensive point mutagenesis of the ISRE demonstrated that the binding specificity of only this protein correlated with ISG expression (Kessler et al. 1988a; Levy et al. 1988a). Furthermore, activation of ISGF3 occurs in IFNα-sensitive cells to an extent commensurate with the degree of transcriptional induction of ISGs, but it is not detected in cells resistant to IFNα (Kessler et al. 1988b; D.E. Levy et al., unpubl.). Thus, the signal transduction pathway initiated by IFNα interaction with its cell-surface receptor involves activation of a latent precursor in ISGF3. In this paper we provide evidence that latent ISGF3 resides in the cytoplasm of uninduced cells where it is activated through a series of at least three steps initiated by IFNα treatment. An undefined modification of one component of ISGF3, stimulated by IFNα-receptor interaction, leads to association with a second polypeptide in the cytoplasm, which is followed by translocation of the active ISGF3 complex to the nucleus. The time course of these events in vivo correlates with the appearance and disappearance of nuclear ISGF3 and with the transcriptional cycle of ISGs. We reconstituted the second step in vitro, a combination of two factors to form active ISGF3, from cytoplasmic extracts of appropriately treated cells.

Results

ISGF3 appears in the cytoplasm of IFNα-treated cells

We used gel retardation analysis (Fried and Crothers 1981; Garner and Revzin 1981; Levy et al. 1988a) to detect ISRE binding factors in IFNα-treated cells using a labeled DNA fragment that contained the ISRE from an ISG promoter. ISGF3 was detected in nuclear extracts (Levy et al. 1988a) prepared from IFNα-treated cells with a time course comparable to the transient nature of ISG transcription (Fig. 1A). Although no ISGF3 binding activity was detectable in extracts from uninduced cells (lane 1), abundant ISGF3 was observed after 30 min to 2 hr of IFNα treatment (lanes 2–4), but had disappeared by 6.5 hr (lane 5), a time when ISG transcription had returned to basal levels (Larner et al. 1986). Although no ISRE-specific DNA-binding activities were detectable in cytoplasmic fractions of untreated cells (data not shown), ISGF3 was detected readily in the cytoplasm after 30 min of IFNα treatment (Fig. 1B). We have shown previously that the entire ISRE sequence is required for full ISG transcription and for ISGF3 binding. This binding specificity differs from that of ISGF1 or ISGF2, nuclear factors whose recognition is limited to the central core sequence of the ISRE (Kessler et al. 1988a). Therefore, differential competition by core and full-length ISRE oligonucleotides provided an assay for ISGF3-specific binding. All specific DNA–protein complexes, including ISGF3, were competed by a full-length, unlabeled ISRE oligonucleotide (lanes 3 and 6). However, the binding of cytoplasmic and nuclear ISGF3 was not competed by oligonucleotides that contained non-specific sequences (lanes 1 and 4) or by oligonucleotides that contained only the central core of the ISRE (lanes 2 and 5). In contrast, the binding of nuclear ISGF1 and ISGF2 was competed effectively by the core sequence (lane 5). Thus, both cytoplasmic and nuclear ISGF3 displayed the same sequence specificity, matching the genetically defined requirements for ISG *trans*-activation (Kessler et al. 1988a).

Active ISGF3 accumulates first in the cytoplasm and later in the nucleus

The detection of active ISGF3 in the cytoplasm of IFNα-treated cells suggested that the active factor might appear in the cytoplasm of treated cells prior to nuclear accumulation. Because ISG transcription is detectable within minutes after IFNα treatment, cytoplasmic and nuclear extracts were prepared after brief exposure of cells to IFNα (Fig. 2A). ISGF3 was detected in cytoplasmic extracts prepared within 1 min of IFNα treatment, increased in extracts made after 5 min, and detectable in extracts prepared after 60 min (lanes 1–6). In contrast, nuclear extracts displayed no ISGF3 activity until 5 min of IFNα treatment (lane 10), reaching maximal levels only after 15 min (lane 11). These extracts were prepared by mechanically shearing cells suspended in hypotonic salt solutions. An S100 fraction obtained by centrifugation of the postnuclear supernatant contained all cytoplasmic ISGF3, suggesting that proteins with this binding activity are freely soluble in crude cytoplasm and are not associated with particles or membranes. Nuclear ISGF3, on the other hand, is not removed from nuclei by additional washes with hypotonic buffer, but is recovered by extraction in 0.3 M NaCl. The differential time of appearance and distinct patterns of

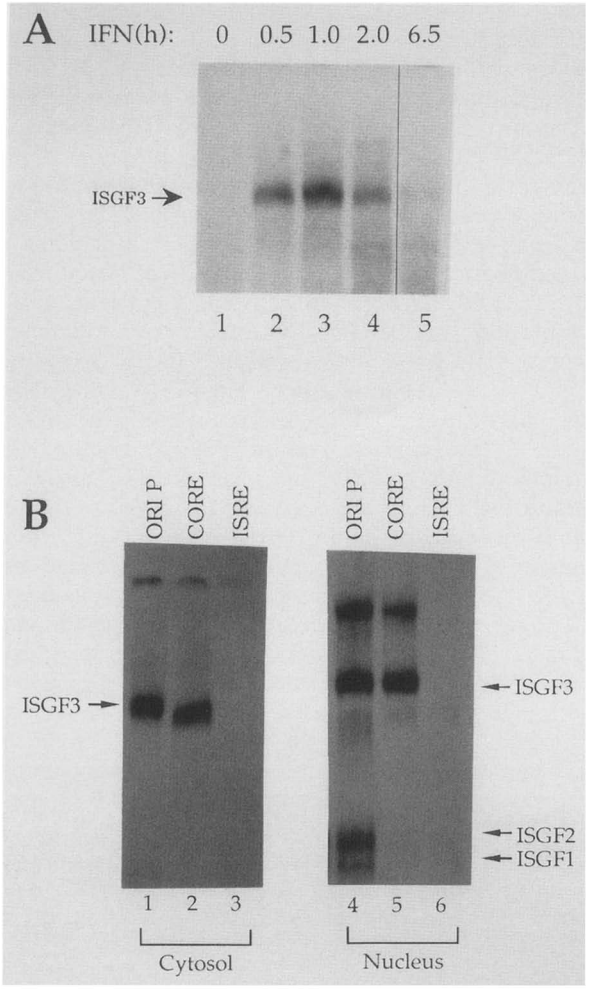

Figure 1. ISGF3 is induced by IFNα in both cytoplasm and nucleus. (*A*) Nuclear extracts of human fibroblasts treated with IFNα for the indicated times were analyzed by gel retardation with a labeled ISRE as a probe. (*B*) Nuclear and cytoplasmic extracts from HeLa cells primed with IFNγ for 16 hr and treated with IFNα for 15 min were subjected to competition gel retardation analysis. The slower mobility complex present in lanes *4* and *5* is observed with extracts that contain high amounts of ISGF3 (either nuclear or cytoplasmic; e.g., see Fig. 2B) and may result from dimer formation. The sequence of the double-stranded oligonucleotide that contained an ISRE core equivalent was 5′-AGGAATTTCCCACTTTCACTTCTC-3′ and its complement; the ISRE oligonucleotide sequence was 5′-GGCTTCAGTTTCGGTTTCCCTTTCCCGAG-3′ and its complement.

extractability of cytoplasmic and nuclear ISGF3 imply that the cytoplasmic form is not the result of artifactual leakage from the nucleus.

We found previously that IFNγ pretreatment of HeLa cells results in higher maximum transcriptional activity after the addition of IFNγ (D.E. Levy et al., unpubl.). We inferred that this IFNγ priming effect resulted from the formation of a large pool of latent ISGF3 that could be converted into the active DNA-binding form after cell treatment with IFNα. We tested the effect of IFNγ priming on the kinetics of cytoplasmic and nuclear ISGF3 accumulation (lanes 13–24). Although much higher levels of ISGF3 were detected (the autoradiograph for lanes 13–24 was exposed for one-sixth as long as that for lanes 1–12), the initial cytoplasmic appearance and the time course of cytoplasmic and nuclear ISGF3 accumulation were identical to those detected in cells treated with IFNα alone.

The temporal lag between cytoplasmic and nuclear accumulation of ISGF3 suggested that cytoplasmic ISGF3 might be a precursor to the nuclear form. In other experiments directed at understanding the biochemical basis of the IFN-induced signal pathway, we tested many pharmacological inhibitors for effects on the IFN response. Of many agents tested, only NaF had an inhibitory effect on ISG expression, reducing ISG54 expression to ~20% of the fully induced levels (data not shown). Therefore, we tested this agent for effects on cytoplasmic and nuclear accumulation of ISGF3. As shown in Figure 2B, 10 mM NaF inhibited the accumulation of nuclear ISGF3 in response to IFNα significantly (cf. lane 8 with lane 6), even in cells pretreated with IFNγ to induce very high levels of this DNA-binding activity. Normalizing to the level of the constitutive activity of ISGF1, NaF reduced nuclear accumulation of ISGF3 by fivefold. In contrast, the corresponding cytoplasmic extracts showed two- to threefold increased levels of ISGF3 after IFNα treatment in the presence of NaF (cf. lane 4 with lane 3). Therefore, the inhibitory effect of NaF on ISG expression is not attributable to an absence of the initial activating signal generated by IFNα receptor occupation or to an inability to activate ISGF3, but results from a decrease in translocation of this factor to the nucleus.

The biochemical basis for this inhibition is not known. Fluoride is known to form AlF_4 in the presence of trace aluminum, a compound that structurally mimics the γ-phosphoryl group of nucleotide triphosphates and exhibits properties resembling nonhydrolyzable analogs of GTP (Sternweis and Gilman 1982). However, we found no evidence for participation of GTP-dependent or other nucleotide triphosphate-dependent proteins in IFNα-stimulated transcription using known inhibitors and activators of G-protein-, kinase-, or adenylate cyclase-coupled processes (Larner et al. 1984; D. Lew et al., unpubl.). The NaF block of ISGF3 nuclear localization is compatible with inhibition of some process that requires shuttling of phosphoryl groups. In this context, we found that cytoplasm to nuclear transit of ISGF3 also is inhibited at low temperatures, requiring several hours instead of minutes at 15°C or less, results compatible with an energy requirement for translocation.

Complementation of cytoplasm from IFNγ-treated cells with cytoplasm from IFNα-treated cells reconstitutes ISGF3 activation in vitro

If ISGF3 were, in fact, activated in the cytoplasm from latent precursors, we hoped to find conditions for reproducing this activation in vitro. As discussed earlier, we found previously that IFNγ priming of HeLa cells ap-

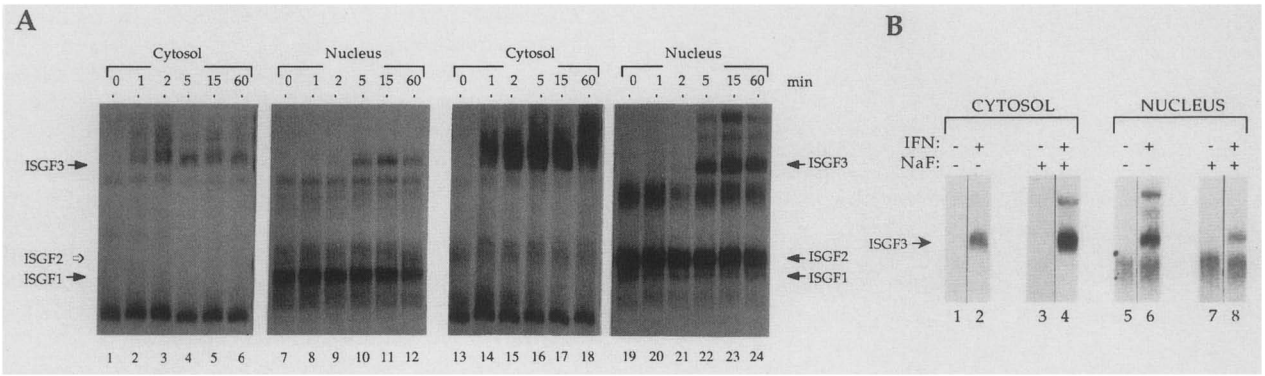

Figure 2. (*A*) ISGF3 appears in the cytoplasm before it is translocated to the nucleus. Cytoplasmic and nuclear extracts of HeLa cells were prepared after IFNα treatments for the indicated times. (Lanes *1–12*) Results from cells treated only with IFNα are shown; (lanes *13–24*) extracts were derived from cells pretreated with IFNγ for 18 hr. The positions of ISGF1 and ISGF2 from nuclear extracts and ISGF3 from nuclear and cytosolic extracts are indicated (arrows). ISGF2 is present only in nuclear extracts from IFNγ-treated cells because the IFNα treatments were too short to allow accumulation of this factor (Levy et al. 1988a). (*B*) NaF inhibits nuclear translocation of ISGF3. HeLa cells pretreated for 18 hr with IFNγ and treated for 15 min with IFNα and 10 mM NaF, as indicated, were tested for the presence of cytoplasmic and nuclear ISGF3.

pears to result from synthesis of high levels of latent ISGF3. We reasoned that extracts of IFNα-treated cells might be capable of activating this latent form of ISGF3 from IFNγ-treated cells.

Therefore, we mixed cytoplasmic extracts from untreated, IFNα-treated, and IFNγ-treated cells, and quantitated the resulting levels of ISGF3 by gel retardation (Fig. 3). No cytoplasmic ISGF3 was detected in extracts from untreated (lane 1) or IFNγ-treated cells (lane 3), and, because limited amounts of extracts were used, little ISGF3 was detectable in IFNα cytoplasm (lane 2). However, when cytoplasmic extracts from IFNα-treated cells were mixed with extracts from IFNγ-treated cells, high levels of ISGF3 were produced (lane 6). These levels of ISGF3 were comparable to the high level detected in extracts from IFNγ-primed cells after IFNα treatment in vivo. This in vitro-produced activity displayed legitimate ISGF3 binding specificity as demonstrated by differential competition with core (lane 7) and full-length (lane 8) ISRE oligonucleotides. A low but detectable level of ISGF3 also was produced by mixing IFNα cytoplasm with cytoplasm from untreated cells (lane 4), indicating that, as expected, untreated cells contain only modest levels of unconverted, latent ISGF3. No ISGF3 was produced by mixing untreated cytoplasm with IFNγ-treated cytoplasm (lane 5); neither of these preparations would be expected to contain a necessary IFNα-activated signal.

Active ISGF3 is formed from distinct protein components that act stoichiometrically

We attempted to find agents or treatments capable of selectively inhibiting or activating in vitro formation of ISGF3. NaF and decreased temperature in vivo inhibited nuclear ISGF3 accumulation and ISG expression. However, neither incubation at subphysiological temperature nor inclusion of NaF in the in vitro reaction inhibited formation of ISGF3 using cytoplasmic extracts from IFNα- and IFNγ-treated cells. Similarly, a large number of other pharmacological agents tested at concentrations known to affect kinases, phosphatases, and G-proteins had no effect on ISGF3 formation in vitro (Table 1) just as they had no effect in vivo (data not shown). Susceptibility of in vitro ISGF3 formation to protease treatment of cytoplasmic extracts indicated that protein components from both IFNα- and IFNγ-treated cells are necessary for ISGF3 formation in vitro (Fig. 3B).

Protein alkylation was found to inhibit ISGF3 activity and in vitro formation (Fig. 3C). Nuclear or cytoplasmic extracts were treated with 10 mM *N*-ethyl maleimide (NEM) under conditions that result in alkylation of reduced cysteine residues, and then dithiothreitol (DTT) was added to inactivate any remaining NEM. This treatment effectively eliminated the ability of ISGF3 formed in vivo to bind DNA (lanes 9–12). In addition, NEM treatment inactivated a cytoplasmic component from IFNγ cells that was necessary for ISGF3 formation in vitro (lane 5). However, it had no effect on necessary components from IFNα-treated cell extracts (lane 4). These results indicated that at least two different proteins, one present in IFNα-treated cells (termed ISGF3α component) and one present in increased amounts in IFNγ-treated cytoplasm (termed ISGF3γ component), were involved in ISGF3 formation. Because of its NEM sensitivity, we suspected that at least the IFNγ-inducible component is part of nuclear ISGF3 and that it is the inactivation of this component by NEM that results in loss of ISGF3 binding activity.

We exploited the NEM sensitivity of mature ISGF3 and of the ISGF3γ component to investigate the stoichiometry and kinetics of ISGF3 formation in vitro (Fig. 4A). Cytoplasmic extracts from IFNα-treated cells were treated with NEM and quenched with DTT to deplete ISGF3 activated in vivo but to retain active ISGF3α. Constant amounts of this NEM-treated extract (lane 1) were mixed with increasing amounts of cytoplasmic protein from nontreated or from IFNγ-treated cells. Low

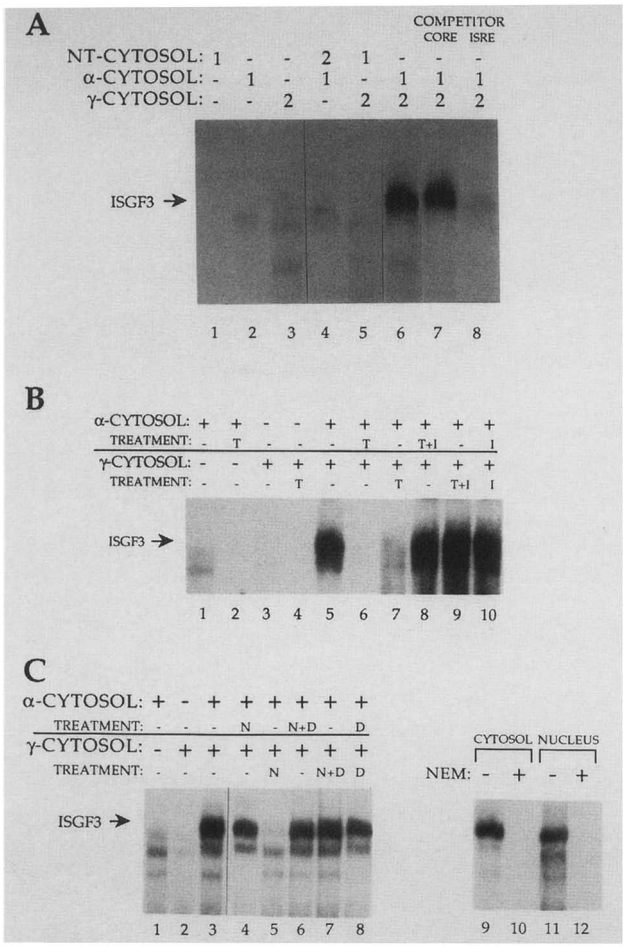

Figure 3. Activation of ISGF3 in vitro. (*A*) Cytoplasmic extracts from HeLa cells treated with IFNα for 1 hr or with IFNγ for 18 hr were mixed in gel retardation binding reactions in the indicated ratios. One unit of cytosol was 5 μg of protein. Nonspecific (lanes *1–6*), core (lane *7*), and full-length ISRE (lane *8*) oligonucleotide competitors were added to the reactions, demonstrating legitimate ISGF3 specificity of the in vitro-activated factor. (*B*) Cytosolic extracts from IFNα- (5 μg) or IFNγ- (10 μg) treated HeLa cells were treated with 4 μg of trypsin for 60 min at room temperature, followed by the addition of 4 μg of bovine pancreatic trypsin inhibitor (T), with trypsin premixed with inhibitor (T + I), or with inhibitor alone (I), as indicated, and assayed by mixing for in vitro activation of ISGF3. The presence of trypsin inhibitor caused a slight alteration in the mobility of the ISGF3–DNA complex. (*C*) Cytoplasmic extracts from IFNα- and IFNγ-treated HeLa cells (5 and 10 μg, respectively) were treated with 10 mM NEM for 10 min at room temperature followed by the addition of 10 mM DTT (N), with 10 mM NEM plus 10 mM DTT (N + D), or with 10 mM DTT alone (D), and then mixed in binding reactions and assayed for ISGF3 (lanes *1–8*). Cytosolic (25 μg) and nuclear (5 μg) extracts from HeLa cells pretreated with IFNγ and then induced with IFNα for 15 min were treated in vitro with 10 mM NEM followed by 10 mM DTT as indicated and assayed for ISGF3 (lanes *9–12*).

but linearly increasing amounts of ISGF3 were formed in response to increasing amounts of cytoplasm from untreated cells (lanes 2–7), whereas much higher and again linearly increasing amounts were formed after addition of increasing extract from IFNγ-treated cells. Similarly, a linear increase in ISGF3 formation was observed in the converse titration when a constant amount of extract from IFNγ-treated cells was mixed with increasing amounts of cytoplasm from IFNα-treated cells (lanes 8–20 and quantitated in Fig. 4B). Although we had hypothesized initially that ISGF3 formation resulted from some enzymatic action of an IFNα-activated component on a protein substrate from the IFNγ-induced cytoplasm, the direct dependence of product on the starting concentration of either component suggested that both are participating stoichiometrically in product formation rather than either one functioning catalytically. The linearity and approximately equal slopes of product formation in response to the addition of either component, and the lack of effect of standard inhibitors (Table 1), are best explained by subunit association to form an active complex.

The apparently nonenzymatic nature of ISGF3 formation in vitro prompted an examination of the temperature and kinetic parameters of the reaction. Varying reaction temperatures over the range from 4°C to 30°C had no effect on in vitro ISGF3 formation (data not shown). Likewise, no effect of increased time of incubation was observed. We determined previously that optimal binding of ISGF3 to DNA in vitro requires 20-min incubations with probe (D.S. Kessler et al., unpubl.). No increase in ISGF3 formation was detected following incubation times longer than the standard 20 min (Fig. 4C). The levels of ISGF3 formed after 30-min (lanes 2 and 5) or 60-min (lanes 3 and 6) preincubations were equal to those observed when no preincubations were allowed. This result was obtained with various starting concentrations of extract. Thus, neither component could act catalytically on excess amounts of the other to produce increased levels of ISGF3. These results are not explained adequately by the hypothesis that there exists a

Table 1. *Agents that failed to affect the cell-free formation of ISGF3*

Treatment	Concentration
H7	45 μM
H8	45 μM
HA1004	45 μM
2-Aminopurine	45 μM
EDTA	5 mM
EGTA	5 mM
NaPPi	2 mM
NaF	10 mM
Sodium vanadate	0.5 mM
NP-40	1%
Formamide	8%
AMP-PNP	5 mM
GMP-PNP	5 mM
GTPγS	5 mM
Hexokinase + glucose	0.1 mg/ml + 10 mM
PMSF	0.5 mM
Aprotinin	3 μg/ml
Leupeptin	0.5 μg/ml
Pepstatin	1.5 μg/ml
Bovine pancreatic trypsin inhibitor	0.4 μg/ml

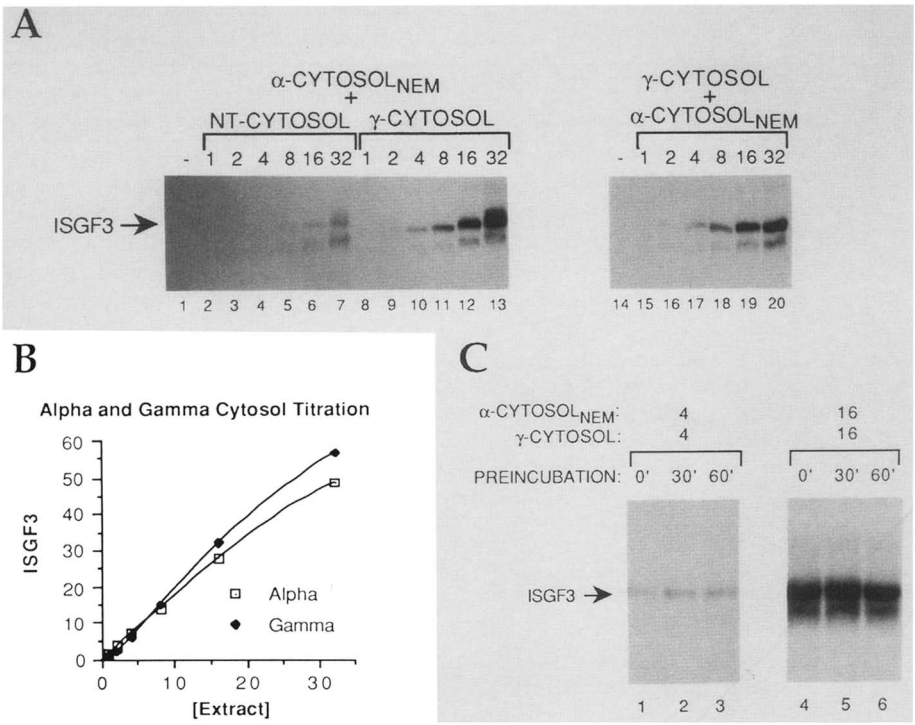

Figure 4. Stoichiometry of in vitro activation of ISGF3. (*A*) Cytoplasmic extracts from HeLa cells treated for 15 min with IFNα were treated in vitro with 10 mM NEM followed by 10 mM DTT to inactivate ISGF3. (Lane *1*) A total of 4.5 μg of this extract was added to increasing amounts of cytoplasm (in arbitrary units equal to ~0.56 μg protein) from nontreated (lanes *2–7*) or from IFNγ-treated cells (lanes *8–13*) in two-fold increments from 0.56 μg (1 unit) to 18 μg (32 units); 4.5 μg of cytoplasm from IFNγ-treated cells (lane *14*) was mixed with similar amounts of NEM-treated cytoplasm from IFNα-treated cells (lanes *15–20*). In vitro activation of ISGF3 was determined by gel retardation following a 20-min incubation at room temperature. (*B*) The autoradiograph shown in *A* was quantitated by densitometry for lanes *8–13* (filled symbols) and for lanes *15–20* (open symbols). (*C*) Cytoplasmic extracts from IFNα- and IFNγ-treated cells were mixed and incubated at room temperature in the absence of labeled DNA for the indicated times followed by addition of probe and a 20-min binding reaction. The reactions contained 2.25 μg (lanes *1–3*) or 9 μg (lanes *4–6*) of each extract, as indicated by units as in *A*.

rapidly decaying catalytic component because preincubation of the separate extracts prior to mixing led to no signficant loss in activity (data not shown). We conclude that ISGF3 is an active DNA-binding complex formed from the stoichiometric association of ISGF3α and ISGF3γ components.

Rapid activation of ISGF3α component in vivo

The association of two precursor components to form active ISGF3 suggested that one result of IFNα action on cells is the conversion of a precursor of ISGF3α component to a form capable of associating with ISGF3γ component. Given the rapidity of IFNα action in vivo (see Fig. 2), we tested the time course of activation of the ISGF3α component of the cell-free reaction. HeLa cells were treated with IFNα for varying lengths of time, from 15 sec to 4 hr, cytoplasmic extracts were prepared, and endogenously produced ISGF3 was inactivated with NEM. The presence of active ISGF3α was determined by the addition of excess cytoplasm from IFNγ-treated cells, followed by gel retardation analysis (Fig. 5A). Active ISGF3α component was detectable after 30 sec of IFNα treatment (lane 4) and reached maximal levels after 5–15 min (lanes 7 and 8). Increased time of IFNα treatment resulted in gradually decreasing levels of ISGF3α component capable of forming ISGF3 in vitro (lanes 9–12), although residual levels were still detectable after 4 hr. These in vitro data are consistent with the time course of accumulation of cytoplasmic ISGF3 in vivo (cf. Fig. 5 with Fig. 2) and indicate that the rate of the ISGF3 formation in vivo is determined by the rate at which ISGF3α component is activated in response to IFNα.

Discussion

We presented evidence that the transcriptional activator for ISGs is derived from latent components resident in the cytoplasm prior to stimulation. IFNα occupation of its cell-surface receptor initiates a series of steps that result in the association of two cytoplasmic polypeptides to produce a complex capable of translocating to the nucleus and binding to DNA (Fig. 6). We reconstituted one step in vitro by mixing cytoplasmic extracts from IFNα-treated cells with extracts from untreated or IFNγ-treated cells. The stoichiometry, kinetics, and pharmacology of this reaction in vivo and in vitro are all consis-

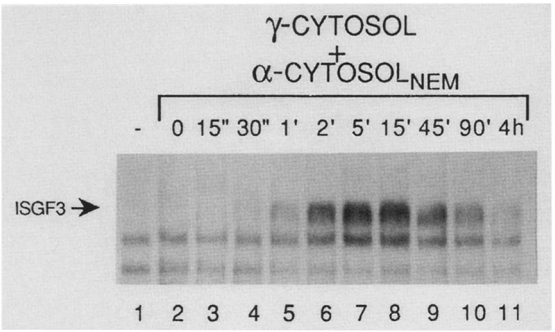

Figure 5. Time course of ISGF3α component activation in vivo. Cytoplasmic extracts were prepared from HeLa cells treated with IFNα for the indicated times and were treated with NEM to inactivate ISGF3. These extracts were mixed with cytoplasm from IFNγ-treated cells and assayed for ISGF3 activation.

tent with the participation of two protein components. The two polypeptides display distinct sensitivities to alkylation by NEM. In addition, we are purifying these activities presently and have separated them chromatographically.

By our model, both proteins preexist in cytoplasm of untreated cells. One (ISGF3γ) is of low abundance in HeLa cells but accumulates to high levels after IFNγ induction. The other (ISGF3α) is modified directly or indirectly in response to IFNα treatment allowing it to associate with ISGF3γ to form active ISGF3, which translocates to the nucleus and binds the ISRE of responsive genes. Recently, we purified nuclear ISGF3 by oligonucleotide affinity chromatography (X. Fu et al., unpubl.). Both ISGF3α and ISGF3γ complementing activities were present in highly enriched fractions of the mature factor, suggesting that the nuclear factor is composed of these two components.

Although the accumulation of ISGF3γ component in response to IFNγ appears to be a peculiarity of HeLa cells (e.g., it is constitutively abundant in diploid fibroblasts), the cytoplasmic activation followed by nuclear accumulation of ISGF3 in response to IFNα also has been observed in FS2 fibroblasts and in Daudi lymphoblastoid cells (data not shown). In addition, while this work was in progress, Dale et al. (1989a) reported a soluble DNA-binding activity induced by IFNα in human fibroblasts and B lymphocytes. Although the DNA binding specificity of this factor was not determined, it might well be equivalent to ISGF3. Most important, this group reported that in vivo activation of this factor occurred in enucleated cytoplasts, indicating that its presence in the cytoplasm is unlikely to result from an artifact of cell fractionation.

Theme and variations in transcription factor activation

The proposed mechanism for ISGF3 activation is a simple solution to the problem of how a cell-surface receptor can mediate selective transcriptional stimulation of a limited set of genes. In other cases, cell-surface signaling appears to involve traditional second messengers. It remains to be determined how perturbations of the cytoplasmic levels of small molecules such as Ca^{2+}, diacylglycerol, inositol polyphosphates, or cAMP that in turn modulate activities of general protein kinases are coupled faithfully to activation of defined gene sets, retaining the fidelity of the receptor–ligand specificity that initiated the intracellular signal. On the other hand, if the transcription factor itself, in some form, is the second messenger then the selectivity of activation is simplified to a single recognition step between receptor and factor in the cytoplasm. It is tempting to speculate that one component of ISGF3 (most likely ISGF3α) actually interacts directly with the IFNα cell-surface receptor and is modified or released in response to IFNα treatment. A general theme of the involvement of protein–protein interaction in cytoplasmic transcription factor activation becomes evident by comparing this proposed mechanism of ISGF3 formation with other signal transduction systems. For example, the glucocorticoid receptor is a silent transcription factor in the cy-

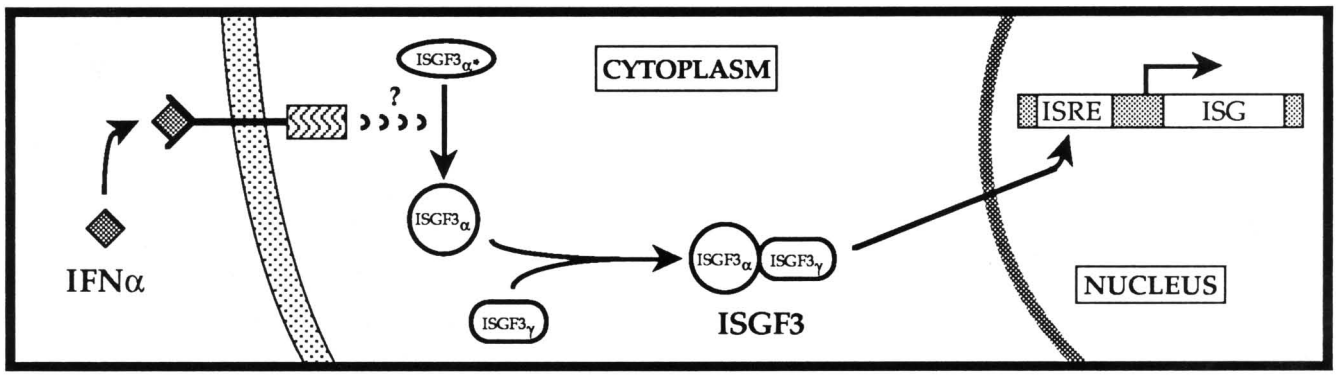

Figure 6. Schematic representation of ISGF3 activation. The mature, DNA-binding form of ISGF3 detected in nuclei of IFNα-treated cells is derived from two latent components preexisting in the cytoplasm, ISGF3α and ISGF3γ. The abundance of ISGF3γ increases in response to IFNγ; ISGF3α changes from an inactive form (ISGF3$_\alpha$*) to an active form through an uncharacterized modification in response to IFNα. This modified form of ISGF3α associates with ISGF3γ, and this complex translocates to the nucleus and binds ISRE sequences of IFN-stimulated genes.

toplasm of uninduced cells. Steroid binding causes the hormone–receptor complex to translocate to the nucleus and bind specific target sequences in responsive genes (Yamamoto 1985; Picard and Yamamoto 1987). It has been shown recently that the silent glucocorticoid receptor is associated with hsp90 in the cytoplasm (Sanchez et al. 1985) and that dissociation of this complex is triggered by steroid binding (Sanchez et al. 1987; Denis et al. 1988; Pratt et al. 1988). Thus, steroid activation of its intracellular receptor also involves protein–protein interactions, although it is release from a complex rather than complex formation that allows nuclear translocation.

Baeuerle and Baltimore (1988a) described another example of cytoplasmic activation of a transcription factor, involving dissolution of a protein–protein complex. A silent precursor to NF-κB is complexed with a specific inhibitor in the cytoplasm of cells not expressing κ immunoglobulin light chain. Upon treatment of cells with bacterial lipopolysaccharide or phorbol esters, the cytoplasmic complex dissociates, allowing active NF-κB to migrate to the nucleus and bind DNA target sequences (Baeuerle and Baltimore 1988b). It is interesting that NF-κB presumably can be activated through a classical second messenger pathway (i.e., protein kinase C). In distinction from the activity of ISGF3, this transcription factor has been implicated in the expression of a growing number of diverse genes in a variety of cell types [e.g., κ light chain, interleukin-2 receptor α-chain, the human immunodeficiency virus LTR, human IFNβ, and serum amyloid protein stimulated by lipopolysaccharide (LPS), dsRNA, and TNF (Sen and Baltimore 1986; Bohnlein et al. 1988; Leung and Nabel 1988; Edbrooke et al. 1989; Lenardo et al. 1989; Visvanathan and Goodbourn 1989)].

A similar mechanism may be employed in the regulation of human *hsp70* gene expression. Again, a sequence-specific DNA-binding protein is activated from a silent cytoplasmic precursor, this time in response to elevated temperature (Larson et al. 1988). Although involvement of a second polypeptide as either inhibitor or activator has not been demonstrated, this activation is also a two-step process of modification or conformation change coupled with nuclear translocation.

Protein–protein associations have been shown to affect the activity of many other DNA-binding proteins. For example, the proto-oncogene products c-*fos* and c-*jun* are nuclear proteins that display weak DNA-binding activity. However, as heterodimers with each other (Chiu et al. 1988), or possibly with other related proteins (Nakabeppu et al. 1988), these complexes bind DNA with specificity and high affinity, correlating with efficient *trans*-activation of specific target genes (Halazonetis et al. 1988; Kouzarides and Ziff 1988; Rauscher et al. 1988). Although the association of this family of transcription factors has not been shown to be modulated in response to any extracellular signal, the activation of ISGF3 could be similar to this fundamental mechanism for creating a high-affinity DNA-binding protein. We suggest that for the case of specific and limited induction of transcription from cell-surface receptors, the cytoplasmic activation of precursors to nuclear DNA-binding proteins may prove to be a general mechanism for assuring an accurate and rapid response.

Materials and methods

Cell culture

Cultured HeLa S3 cells obtained from ATCC (Rockville, Maryland) were maintained in Joklik's modified Eagle's medium supplemented with 5% calf serum and antibiotics. Human diploid fibroblasts (FS2) were obtained from E.K. Knight and E.I. duPont and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics. For IFN treatments, human recombinant bacteria-derived IFNα, a kind gift of Dr. P. Sorter (Hoffman-LaRoche, Nutley, New Jersey), was added to culture media at 15 pM (500 U/ml) unless noted otherwise; human recombinant bacteria-derived IFNγ, generously provided by Dr. D. Vapnek (Amgen, Thousand Oaks, California), was used at 60 pM (20 U/ml).

Cell extracts

Nuclear and cytoplasmic cell extracts were prepared by a modification of the procedure of Dignam et al. (1983) as described previously (Levy et al. 1988a). Cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS), and lysed by homogenization in hypotonic buffer A [20 mM HEPES (pH 7.0), 10 mM KCl, 1 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 0.5 mM DTT, and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. Nuclei were separated from cytoplasmic extracts by centrifugation at 200*g*, washed in buffer A, and centrifuged at 14,000*g* prior to extraction with 0.3 M NaCl in buffer A without detergent. Nuclear extracts were clarified by centrifugation at 14,000*g*; cytoplasmic extracts were centrifuged at 100,000*g*. For some experiments performed on small numbers of cells, cytoplasmic extracts were clarified only at 14,000*g*. No differences were noted between extracts prepared by the two procedures. For time course experiments, cells were resuspended in media at $\sim 5 \times 10^7$ cells/ml and treated with 1.2 nM IFNα (40,000 U/ml) to saturate receptor binding quickly. Cells were shaken at 37°C for the times indicated in the figure legends, and then were quenched with 100 volumes of PBS at 0°C, centrifuged, and extracted as described. It was found that treated cells could remain on ice for up to 4 hr with no change in the amount or cytoplasmic and nuclear distribution of ISGF3 or its subunits (D.E. Levy, unpubl.).

Protein–DNA binding assays

Protein–DNA binding assays were performed by gel retardation (Fried and Crothers 1981; Garner and Rezvin 1981) as described previously (Kessler et al. 1988a; Levy et al. 1988a). Nuclear and cytoplasmic extracts derived from equal numbers of cells were incubated with 5 fmoles of radiolabeled ISG15 promoter fragment containing the ISRE sequence (−115 to −39) in the presence of nonspecific DNA [4 μg of double-stranded poly[d(I-C)], 0.5 μg of plasmid DNA, and 5 mM nucleotide] in a total volume of 12.5 μl (Kessler et al. 1988a). Unless noted otherwise, binding reactions contained 5 μg of nuclear or 25 μg of cytoplasmic protein. Under these conditions, >90% of the labeled probe remained unbound, insuring that activity measurements were made in probe excess. For competition assays, 10 ng of specific or nonspecific double-stranded oligonucleotides were included in each binding reaction. Following 20-min incubations at room temperature, 5 μl of each binding reaction were fractionated on 4.8% polyacrylamide gels run in 20 mM Tris-borate-EDTA for 3 hr at 300 V.

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