Cell Biol. **104**, 331 (1987); J. C. Edmonson, R. K. H. Liem, J. E. Kuster, M. E. Hatten, *ibid.* **106**, 505 (1988); H. Antonicek and M. Schachner, *J. Neurosci.* **8**, 2961 (1988); C.-M. Chuong, *Experientia* **46**, 892 (1990).
5. J. J. LoTurco, M. G. Blanton, A. R. Kriegestein, *J. Neurosci.* **11**, 792 (1991); D. Moran, *Am. J. Anat.* **192**, 14 (1991).
6. Using a serial assay technique, we estimated the average length of the migratory pathway in vitro by measuring the distance from the DiI-labeled cell soma to the border between the external granular and the molecular layers at different time points (Fig. 1). The mean length of the migration route for labeled cells after 2 hours was 33.0 ± 15.9 μm (mean ± SD, n = 151), a rate of 16.5 μm per hour (Fig. 2). The migrating route was 53.5 ± 22.7 μm (n = 136) after 4 hours and 62.5 ± 22.1 μm (n = 136) after 6 hours, which indicated that the speed of migration slows down with time (Fig. 2). To determine whether these measurements reflect granule cell migration in vivo, we next stained migrating granule cells in living animals by applying DiI on the cerebellar surface and then examined their position in fixed tissue with confocal microscopy (MRC-600, Bio-Rad). The mean displacement of granule cells in vivo was 61.2 ± 25.2 μm per 7 hours (n = 122), a distance comparable to that obtained in slice preparations (Fig. 2).
7. G. Fishell and M. E. Hatten, *Development* **113**, 755 (1991).
8. S. Fujita, *J. Cell Biol.* **32**, 277 (1967).
9. S. B. Cull-Candy, C. G. Marshall, D. Ogden, *J. Physiol. (London)* **414**, 179 (1989); P. A. Slesinger and J. B. Lansman, *ibid.* **435**, 101 (1991); C. Marchetti, C. Carignani, M. Robello, *Neuroscience* **43**, 121 (1991).
10. R. W. Tsien *et al.*, *Trends Neurosci.* **11**, 431 (1988).
11. P. Hess, *Annu. Rev. Neurosci.* **13**, 337 (1990); E. Sher and F. Clementi, *Neuroscience* **42**, 301 (1991); W. A. Horne, E. Hawrot, R. W. Tsien, *J. Biol. Chem.* **266**, 13719 (1991); B. M. Olivera, J. Rivier, J. K. Scott, D. R. Hillyard, L. J. Cruz, *ibid.*, p. 22067.
12. P. Forscher, *Trends Neurosci.* **12**, 468 (1989).
13. O. T. Jones, D. L. Kunze, K. J. Angelides, *Science* **244**, 1189 (1989).
14. S. B. Kater and L. R. Mills, *J. Neurosci.* **11**, 891 (1991).
15. S. J. Robson and R. D. Burgoyne, *Neurosci. Lett.* **104**, 110 (1989).
16. J. A. Wagner *et al.*, *J. Neurosci.* **8**, 3354 (1988); N. Martin-Moutot, M. Seagar, F. Couraud, *Neurosci. Lett.* **115**, 300 (1990); A. Vigers and K. H. Pfenninger, *Dev. Brain Res.* **60**, 197 (1991).
17. P. E. Hockberger, H.-Y. Tseng, J. A. Connor, *J. Neurosci.* **7**, 1370 (1987).
18. J. A. Connor, H.-Y. Tseng, P. E. Hockberger, *ibid.*, p. 1384; M. J. Courtney, J. J. Lambert, D. G. Nicholls, *ibid.* **10**, 3873 (1990).
19. J. Holliday and N. C. Spitzer, *Dev. Biol.* **141**, 13 (1990).
20. M. C. Nowycky, A. P. Fox, R. W. Tsien, *Nature* **316**, 440 (1985).
21. C. R. Artalejo, R. L. Perlman, A. P. Fox, *Neuron* **8**, 85 (1992).
22. R. J. Miller, *Science* **235**, 46 (1987).
23. D. F. Newgreen and D. Gooday, *Cell Tissue Res.* **329**, 329 (1985).
24. P. Doherty, S. V. Ashton, S. E. Moore, F. S. Walsh, *Cell* **67**, 21 (1991).
25. This research was supported by the U.S. Public Health Service and by a Human Frontiers grant from Japan. We thank R. Cameron, P. S. Goldman-Rakic, and K. Wikler for useful suggestions and comments on the manuscript.

30 March 1992; accepted 17 June 1992

Interferon-Dependent Tyrosine Phosphorylation of a Latent Cytoplasmic Transcription Factor

Chris Schindler, Ke Shuai, Vincent R. Prezioso, James E. Darnell, Jr.*

The interferon-α (IFN-α)–stimulated gene factor 3 (ISGF3), a transcriptional activator, contains three proteins, termed ISGF3α proteins, that reside in the cell cytoplasm until they are activated in response to IFN-α. Treatment of cells with IFN-α caused these three proteins to be phosphorylated on tyrosine and to translocate to the cell nucleus where they stimulate transcription through binding to IFN-α–stimulated response elements in DNA. IFN-γ, which activates transcription through a different receptor and different DNA binding sites, also caused tyrosine phosphorylation of one of these proteins. The ISGF3α proteins may be substrates for one or more kinases activated by ligand binding to the cell surface and may link occupation of a specific polypeptide receptor with activation of transcription of a set of specific genes.

Many polypeptide growth factors and cytokines induce the transcription of largely nonoverlapping sets of genes (*1–5*). The basis for this high degree of specificity in gene activation by particular polypeptide ligands is not known. Many cell surface receptors have intrinsic biochemical activities that transmit signals to the cell interior (*6–8*). For example the internal domains of the receptors for platelet-derived growth factor and epidermal growth factor (*6*, *8*) have tyrosine kinase activity that is required for their biologic function. However, the receptors for many ligands such as growth hormone, tumor necrosis factor, interleukin-1, IFN-α, and IFN-γ do not have known enzyme activities associated with them (*1–5*). However some of these receptors could have associated kinases such as $p56^{lck}$, which associates with the CD4 portion of the T cell receptor complex (*9*). A number of tyrosine kinase substrates, particularly serine threonine kinases, are targets of cell surface–associated enzymes (*10–13*). However, no substrate has been described that serves to link tyrosine kinase activity at the cell surface to stimulation of transcription in the nucleus (*14*).

We have studied genes whose transcription is stimulated by binding of IFN-α and IFN-γ to their specific cell surface receptors (*1*, *15*, *16*). In the case of IFN-α, the factor responsible for the transcriptional activation is a complex of four proteins termed ISGF3 (interferon-stimulated gene factor 3) (*17–19*). Three of these proteins, collectively termed ISGF3α proteins, reside in the cytoplasm in unstimulated cells (*18*). In cells stimulated with IFN-α, these proteins, which are 113, 91, and 84 kD in size (*19*), are translocated to the nucleus where, together with a 48-kD DNA binding protein (*19*, *20*), they form the ISGF3 complex. ISGF3 binds tightly to a specific DNA sequence, the ISRE, or interferon stimulated response element (*17*, *21*) and directs IFN-α–dependent gene transcription in the nucleus. We have cloned cDNAs that encode the 48-kD DNA binding protein (*22*) and the 113-, 91-, and 84-kD ISGF3α proteins (*23*, *24*) and prepared several different antisera to portions of these proteins.

The NH_2-terminal 701 amino acids of the 91- and 84-kD proteins are identical, and the 91-kD protein contains 38 additional amino acids at its COOH-terminus (*23*). The 113-kD protein is a member of the same gene family as the 91- and 84-kD proteins and shares about 40% amino acid identity with the 91- and 84-kD proteins, but can be distinguished by specific antisera (*24*). Three rabbit antisera were prepared to bacterial fusion proteins and used to examine IFN-induced changes in the abundance of the proteins: antiserum to amino acids 671 to 806 of the 113-kD protein (anti-113), antiserum to amino acids 598 to 705 of the 91-kD protein (anti-91), and antiserum to the COOH-terminal 36 amino acids of the 91-kD protein (anti-91T). Protein extracts from untreated [^{35}S]methionine-labeled cells were used to test each antiserum. Anti-113 precipitated only the 113-kD protein (Fig. 1A). Anti-91 precipitated both the 91- and 84-kD proteins (Fig. 1B) and anti-91T precipitated only the 91-kD protein (Fig. 1B). Thus, in untreated cells the three proteins appear not to be associated with one another.

Extracts of HeLa cells treated with IFN-α, IFN-γ alone, or IFN-α after prior treatment with IFN-γ were examined. IFN-α alone activates ISGF3, but IFN-γ alone does not (*18*, *19*). However, treatment of HeLa cells with IFN-γ for 16 to 18 hours renders them ten times more responsive to IFN-α than untreated cells and ten times more ISGF3 is formed (*25*). This enhanced response of HeLa cells is due at least in part to an increased amount of the

Laboratory of Molecular Cell Biology, The Rockefeller University, New York, NY 10021.

*To whom correspondence should be addressed.

48-kD DNA-binding protein, ISGF3γ. Immunoprecipitation of extracts from [^{35}S]methionine-labeled cells showed that prolonged treatment with IFN-γ also increased the amounts of the 113-, 91- and 84-kD proteins (Fig. 1, A and B). Thus, superinduction of ISGF3 in these cells results from an increase in the abundance of all four components of ISGF3. Treatment with IFN-γ alone left each protein unassociated with the other proteins; the anti-113 precipitated only the 113-kD protein and anti-91 precipitated only the 91-kD protein (Fig. 1A).

After treatment of HeLa cells with IFN-α (6 min) the three ISGFα proteins appeared to form a complex. In extracts from cells treated with IFN-α alone, anti-113 precipitated both the 91- and 84-kD proteins as well as the 113-kD protein (Fig. 1A). When the three proteins were immunoprecipitated from cells that had been treated with IFN-γ for 18 hours, the effect of the brief IFN-α treatment on apparent complex formation was clearer. Under these conditions anti-113 precipitated more of the labeled 113-kD protein and more of the 91- and 84-kD proteins as well (Fig. 1A). A similar amount of immunoprecipitable complex was recovered from cells treated with IFN-α for 6 or 25 min (Fig. 1A). We have not been able to precipitate all three proteins with the available antisera to the 91-kD protein. The epitopes of the 91-kD protein might be inaccessible within a protein complex. These results suggest that the 113-, 91-, and 84-kD proteins are separate in the cytoplasm before activation and that during cytoplasmic activation a change in structure of the individual ISGFα proteins occurs that allows anti-113 to precipitate all three proteins. This probably indicates association between the proteins.

We examined the intracellular distribution of the antibody-reactive ISGFα proteins before and after IFN-α treatment by analysis of immunofluorescence (Fig. 2) (*26*). Untreated HeLa cells or human fibroblasts showed a diffuse cytoplasmic reactivity of the 113- and 91-kD antigens whereas the nuclei appeared to lack any reactivity. In cells that had been treated with IFN-α, however, both the 113- and 91-kD proteins were detected in the nucleus with apparently less protein remaining in the cytoplasm. To facilitate distinction between the nucleus and cytoplasm, we also performed the immunofluorescence experiments on human fibroblasts, which have abundant, well-spread cytoplasm. The IFN-dependent translocation of the 113- and 91-kD proteins (and presumably the 84-kD protein) was more easily visible in these cells. NaF blocks the nuclear translocation but not the cytoplasmic formation of ISGF3 (*18*), and NaF also blocked the IFN-dependent appearance of nuclear antigen detected by anti-113 or anti-91 (*27*).

Because staurosporine, a broad spectrum inhibitor of protein kinases including both tyrosine kinases and protein kinase C (*28*), inhibits transcriptional induction by IFN-α and inhibits ISGF3 formation, we tested whether the ISGF3α proteins were phosphorylated. The ISGF3α proteins were immunoprecipitated from extracts of HeLa cells that had been incubated with [^{32}P]orthophosphate. The immunoprecipitates from cells not treated with IFN-α contained little or no ^{32}P-labeled 113-, 91-, or 84-kD protein, whereas after IFN-α treatment, the precipitates contained ^{32}P-

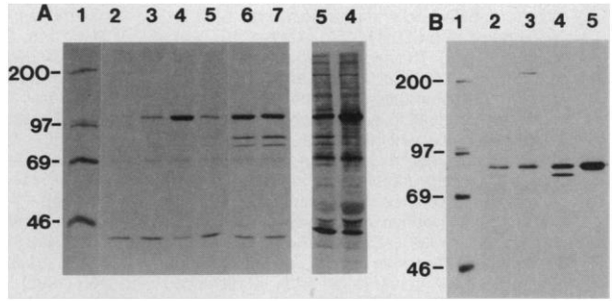

Fig. 1. Immunoprecipitation of ISGF3α proteins. (**A**) [^{35}S]methionine-labeled HeLa cells [1 × 10^7 cells in methionine-free DME (Dulbecco modified Eagle's medium), incubated with [^{35}S]methionine (100 mCi/ml; 1.2 Ci/μmol) for 4 hours] were lysed with NP-40 (*36*) after incubation with or without IFN (IFN-γ, 5 ng/ml for 18 hours, resupplied during labeling; IFN-α, 1000 U/ml for the times indicated). Proteins in extracts were precipitated (*29*) with anti-113 (lanes 3 to 7). Lane 1, ^{14}C-labeled protein size markers (Amersham); lane 2, no IFN treatment, proteins immunoprecipitated with preimmune serum; lane 3, no IFN treatment; lane 4, IFN-γ for 18 hours; lane 5, IFN-α for 25 min; lane 6, IFN-γ for 18 hours and then IFN-α for 6 min; lane 7, IFN-γ for 18 hours and then IFN-α for 25 min. Photo to right shows a longer autoradiographic exposure of lanes 5 and 4. (**B**) Same as (A), but cells were labeled for 3 hours with [^{35}S]methionine (150 mCi/ml) and extracts were precipitated with anti-91 and anti-91T. Lane 1, ^{14}C-labeled markers. Proteins from untreated cells (lanes 2 and 3) or from cells treated with IFN-γ (16 hours) (lanes 4 and 5) were immunoprecipitated with anti-91 (lanes 2 and 4) or anti-91T (lanes 3 and 5).

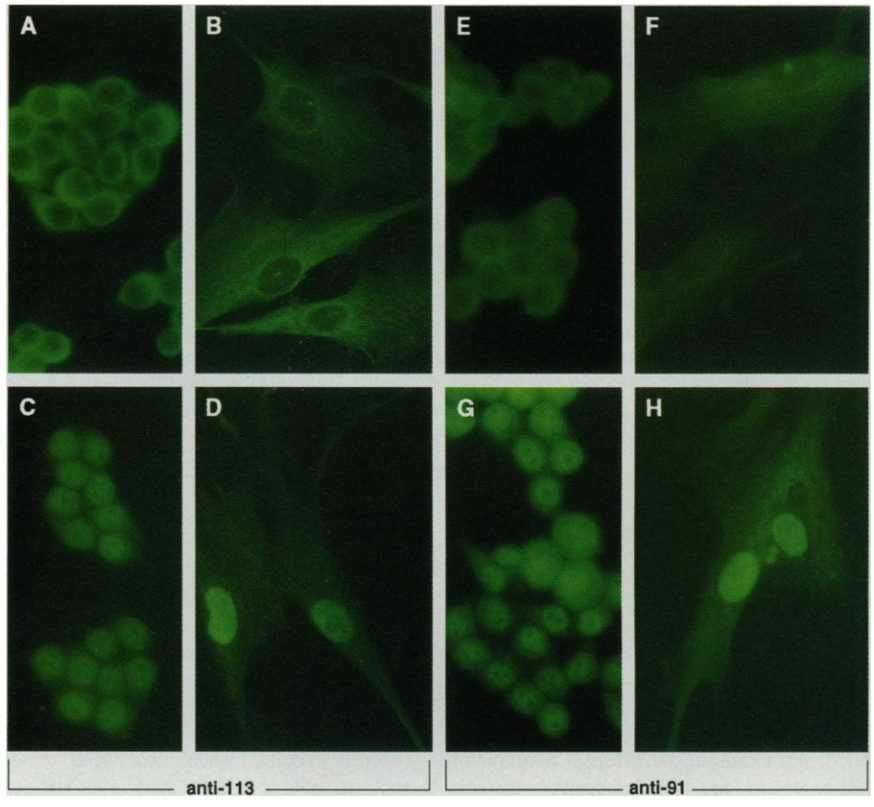

Fig. 2. Transport of the 91- and 113-kD proteins to the nucleus after treatment of cells with IFN-α. HeLa cells (**A, C, E,** and **G**) and human FS2 cells (**B, D, F,** and **H**) were left untreated (A, B, E, and F) or treated with IFN-α (500 U/ml; 30 min) (C, D, G, and H) and were examined by immunofluorescence (*26*) with anti-113 or anti-91T (indicated below figure).

labeled 113- and 91-kD proteins (Fig. 3A). Proteins were also precipitated from extracts of cells that had been treated with IFN-γ to enhance the amount of ISGF-3 formed. The overnight treatment with IFN-γ alone did not result in immunoprecipitation of ^{32}P-labeled proteins with anti-113. However, IFN-α treatment of cells that had already been treated with IFN-γ increased the amount of the ^{32}P-labeled 113- and 91-kD proteins that was precipitated with the antibodies. Moreover, ^{32}P-labeled 84-kD protein was observed in immunoprecipitates from cells that had been treated with IFN-γ and then IFN-α (Fig. 3A).

We also examined the effect of staurosporine on the IFN-α–induced phosphorylation of the ISGF3 proteins. The inhibitor virtually eliminated the incorporation of ^{32}P into the 113-, 91-, and 84-kD proteins (Fig. 3B). Because staurosporine prevents activation of ISGF3α in the cytoplasm, this result suggests that the phosphorylation event might be necessary for IFN-α–dependent formation of ISGF3 and subsequent transcriptional induction. Also, immunofluorescence studies showed that staurosporine-treated cells had no increased nuclear fluorescence from ISGF3 after IFN-α treatment (*27*). The protein kinase inhibitors H7 and 6-aminopurine, which don't affect IFN-α–dependent transcriptional stimulation (*16*), did not affect the phosphorylation of the ISGF3α proteins (Fig. 3).

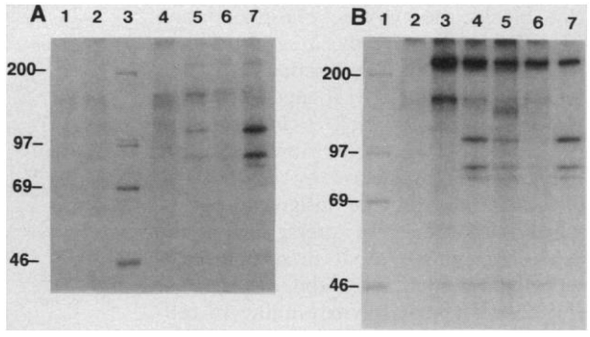

Fig. 3. IFN-α–dependent phosphorylation of 113-, 91-, and 84-kD proteins. HeLa cells (1×10^7) were labeled in phosphate-free DME (supplemented with dialyzed serum) containing [^{32}P]orthophosphate (400 μCi/ml) for 1 hour. After various treatments, proteins from cell extracts were immunoprecipitated with anti-113 or preimmune serum as in Fig. 1. Phosphatase inhibitors (50 mM NaF, 30 mM pyrophosphate, and 0.1 mM Na_3VO_4) were added during extract preparation. (**A**) IFN-α–dependent phosphorylation of ISGF3 proteins. Proteins were immunoprecipitated with preimmune serum (lanes 1 and 2) or anti-113 (lanes 4 to 7). IFN treatments were: lanes 1 and 6, IFN-γ alone (16 hours); lane 5, IFN-α alone (7 min); lanes 2 and 7, IFN-γ followed by IFN-α; lane 4, proteins from untreated cells; and lane 3, protein size markers. (**B**) Inhibition by staurosporine of phosphorylation of ISGF3α proteins. Lane 1, protein size markers. Proteins were precipitated with preimmune serum (lane 2) or anti-113 (lanes 3 to 7). Cells were left untreated (lane 3) or treated with IFN-γ for 16 hours and IFN-α for 7 min (lanes 4 to 7). Cells were treated with the protein kinase inhibitors 2-aminopurine (9 mM) (lane 5), staurosporine (500 μM, Biomol) (lane 6), and H7 (50 mM, Biomol) (lane 7) for 20 min.

To determine the cellular compartment in which phosphorylation of the ISGF3α proteins occurred, we exposed ^{32}P-labeled cells to IFN-α for 3 or 30 min. Separate testing of nuclear and cytoplasmic extracts for phosphorylated ISGF3α proteins demonstrated that most of the immunoprecipitable ^{32}P-labeled 113- or 91-kD protein was in the cytoplasm after 3 min and that the proteins were equally divided between the two compartments after 30 min (*27*). Also, NaF, which allows active ISGF3 to form but prevents its movement to the nucleus, did not block phosphorylation of the ISGF3α proteins (*27*), supporting the conclusion that phosphorylation and activation of ISGF3 are cytoplasmic events.

We determined the amino acid phosphorylated in response to IFN-α in the 113-, 91-, or 84-kD proteins (*29*). In each case the great majority of the ^{32}P was contained in phosphotyrosine (Fig. 4A). The ISGF3α proteins from cells not treated with IFN-α, but incubated with [^{32}P]orthophosphate for longer periods of time, contained phosphoserine (or phosphoserine and a small amount of phosphothreonine) but no phosphotyrosine. In this experiment (Fig. 4) cells were treated with IFN-γ for 16 hours and IFN-α for 7 min. Similar results were obtained for the 113- and 91-kD proteins from cells treated with IFN-α alone (*27*). In fibroblasts, IFN-α alone caused tyrosine phosphorylation of these proteins (*30*).

We digested ^{32}P-labeled 113-, 91-, and 84-kD immunoprecipitated proteins with

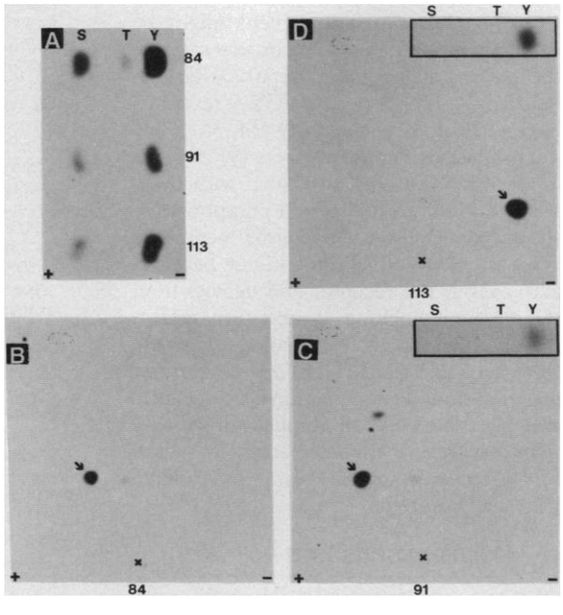

Fig. 4. Phosphoamino acid and phosphopeptide analysis of 113-, 91-, and 84-kD proteins. (**A**) ^{32}P-labeled, immunoprecipitated 84-, 91-, and 113-kD protein bands obtained as in Fig. 3 were eluted, acid-hydrolyzed, and analyzed for the presence of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) (*36*). (**B** to **D**) Two-dimensional phosphopeptide analysis of ^{32}P-labeled immunoprecipitated (B) 84-, (C) 91-, and (D) 113-kD proteins digested with thermolysin. Phosphopeptide analysis was performed essentially as described (*37*). The polarity of electrophoresis was as indicated; "X" marks the origin, and the dashed oval shows the migration of bromphenol red marker. The predominant phosphopeptide is indicated by an arrow, and phosphoamino acid determinations for these recovered proteins are shown in the insets.

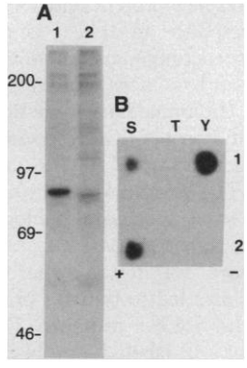

Fig. 5. Phosphorylation on tyrosine of the 91-kD protein in fibroblasts after treatment of cells with IFN-γ. (**A**) Fibroblasts (FS2 cells) were grown in DME and labeled with [^{32}P]orthophosphate as in Fig. 3. The labeled 91-kD protein was immunoprecipitated with anti-91T and subjected to gel electrophoresis. Cells were treated with IFN-γ (5 ng/ml) for 7 min (lane 1) or left untreated (lane 2). (**B**) The darkest bands in the 91-kD region from IFN-γ–treated (lane 1) and untreated cells (lane 2) were recovered and subjected to hydrolysis for phosphoamino acid analysis as in Fig. 4 (Y, phosphotyrosine; T, phosphothreonine; and S, phosphoserine).

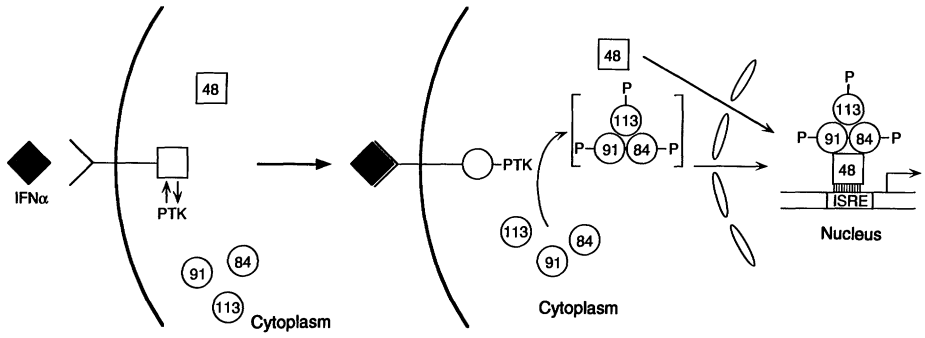

Fig. 6. Diagram of proposed mechanism of activation of ISGF3 by IFN-α. The three ISGF3α proteins of 113, 91, and 84 kD are shown as tyrosine kinase substrates that interact to form a complex. The complex translocates to the nucleus and binds the ISRE. The protein tyrosine kinase (PTK) is shown reversibly associating with the receptor, being present when the receptor is occupied, but its nature and location within the cell are not known.

thermolysin to produce small peptides and then separated the resulting peptides by electrophoresis and chromatography. Autoradiography identified a single major labeled phosphopeptide in each protein (Fig. 4, B through D). The migration of the peptide from the 91- and 84-kD proteins was identical, whereas that from the 113-kD protein differed. Phosphotyrosine was the only labeled amino acid in these peptides (Fig. 4, C and D). These results suggest that a single site in each of the proteins is the target for tyrosine phosphorylation.

We have also studied the transcriptional induction by IFN-γ of the guanylate binding protein (GBP) gene in both fibroblasts and HeLa cells (*16, 31, 32*). The transcriptional response to IFN-γ is sluggish in HeLa cells (10 to 15 hours to reach a maximum) and requires new protein synthesis. However, the response to IFN-γ is prompt in fibroblasts reaching a maximum in less than an hour, and no new protein synthesis is required. The immediate induction of transcription in fibroblasts in response to IFN-γ requires activation of latent cytoplasmic proteins. The protein kinase inhibitors H7 (*16*) and staurosporine also block IFN-γ–dependent transcription (*30*).

Therefore we tested whether IFN-γ treatment also led to tyrosine phosphorylation of the ISGF3 proteins. Proteins in extracts of ^{32}P-labeled fibroblasts treated with IFN-γ for 15 min were immunoprecipitated with anti-113 or anti-91. No IFN-γ–dependent ^{32}P-labeled protein was precipitated by anti-113 (*33*) but anti-91 immunoprecipitated a ^{32}P-labeled 91-kD protein. The ^{32}P was incorporated primarily into phosphotyrosine (Fig. 5). Thus, both IFN-α and IFN-γ stimulated tyrosine phosphorylation of one of the ISGF3 proteins.

The three ISGF3α proteins of 113, 91, and 84 kD are all tyrosine kinase substrates in the cytoplasm of cells treated with IFN-α. Because the kinase inhibitor staurosporine blocks the tyrosine phosphorylation (Fig. 3) and because the drug also blocks IFN-α–dependent transcription and ISGF-3 formation (*28*), it appears that the tyrosine phosphorylation is important in the activation of these proteins in the transcriptional induction by IFN-α. The present results provide insight into how specific ligand-receptor interactions at the cell surface might result in specific transcriptional responses in the cell nucleus (Fig. 6). Whereas IFN-α binding to cells (HeLa cells or fibroblasts) led to immediate phosphorylation of the 113-, 91-, and 84-kD proteins, IFN-γ binding to fibroblasts led to phosphorylation of only the 91-kD protein. IFN-α (*19*) and IFN-γ (*31, 32*) activate transcription from different DNA response elements, which is consonant with the different patterns of tyrosine phosphorylation induced by these polypeptides. Because of the speed of cytoplasmic activation of ISGF3 proteins (*18*), it seems possible that the phosphorylation event might be catalyzed by a kinase on the inner surface of the plasma membrane (Fig. 6). Although neither the IFN-α nor the IFN-γ receptors have intrinsic kinase activity (*34, 35*), they might have *src* family members (*9*) or other types of kinases (*14*) associated with them (*9*) that could recognize and phosphorylate specific substrates at the plasma membrane (Fig. 6). Alternatively, if a kinase becomes activated at the receptor and departs from the plasma membrane, it must carry with it the observed specificity. That is, if a kinase were activated at the IFN-α receptor and departed from it, it would have to recognize and phosphorylate all three ISGF3α proteins, whereas a kinase liberated from the IFN-γ receptor would only phosphorylate the 91-kD protein.

REFERENCES AND NOTES

1. D. Levy and J. E. Darnell, *New Biol.* **2**, 923 (1990).
2. M. E. Greenberg, L. A. Greene, E. B. Ziff, *J. Biol. Chem.* **260**, 14101 (1985).
3. G. H. Murdock, M. G. Rosenfeld, R. M. Evans, *Science* **218**, 1315 (1982).
4. T. H. Lee, S. W. Lee, E. B. Ziff, J. Vilcek, *Mol. Cell. Biol.* **10**, 1982 (1990).
5. W. J. Leonard, M. Krönke, N. J. Peffer, J. M. Dep-pen, W. C. Greene, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6281 (1985).
6. G. Gill, in *Oncogenes and the Molecular Origins of Cancer*, R. A. Weinberg, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990), pp. 67–96.
7. A. Gilman, *Annu. Rev. Biochem.* **56**, 615 (1987).
8. H. D. Bourne and A. L. DeFranco, in *Oncogenes and the Molecular Origins of Cancer*, R. A. Weinberg, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990), pp. 97–124.
9. A. Veillette, M. A. Bookman, E. M. Horak, J. B. Bolen, *Cell* **55**, 301 (1988); C. E. Rudd, J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, S. F. Schlossman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5190 (1988).
10. G. Draetta *et al.*, *Nature* **336**, 738 (1988).
11. G. Carpenter and S. Cohen, *J. Biol. Chem.* **265**, 7709 (1991).
12. T. Hunter, in *Oncogenes and the Molecular Origins of Cancer*, R. A. Weinberg, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1990), p. 142.
13. J. R. Glenny, Jr., *J. Immunochem.* **5**, 1 (1991).
14. M. V. Chao, *Cell* **68**, 995 (1992).
15. A. C. Larner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6733 (1984).
16. D. Lew, T. Decker, J. E. Darnell, Jr., *Mol. Cell. Biol.* **9**, 5404 (1989).
17. D. E. Levy, D. S. Kessler, R. Pine, N. Reich, J. E. Darnell, Jr., *Genes Dev.* **2**, 383 (1988).
18. D. E. Levy, D. S. Kessler, R. I. Pine, J. E. Darnell, Jr., *ibid.* **3**, 1362 (1989).
19. X. Fu, D. S. Kessler, S. A. Veals, D. E. Levy, J. E. Darnell, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8555 (1990).
20. D. S. Kessler, S. A. Veals, X.-Y. Fu, D. E. Levy, *Genes Dev.* **4**, 1753 (1992).
21. N. Reich *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6394 (1987).
22. S. Veals *et al.*, *Mol. Cell. Biol.*, in press.
23. C. Schindler *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
24. X.-Y. Fu *et al.*, *ibid.*, in press.
25. D. E. Levy, D. J. Lew, T. Decker, D. S. Kessler, J. E. Darnell, Jr., *EMBO J.* **9**, 1105 (1990).
26. For immunofluorescence studies, cells were rinsed twice in phosphate-buffered saline (PBS) and fixed in a solution of methanol:acetone (1:1) for 2 min. After two washes with TBST (10 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.02% Tween-20, nonspecific protein adsorption was blocked by incubation of cells for 40 min in TBST containing bovine serum albumin (3%). Anti-113 was diluted 1:100 and anti-91T was diluted 1:500 in blocking buffer and incubated with the fixed cells for 2 hours at 25°C. Cells were washed in TBST, and secondary antibody (fluorescein-conjugated donkey antibody to rabbit immunoglobulin G, diluted 1:200 in blocking buffer) was added to the cells for 1 hour at room temperature. After a final wash in TBST, the cells were mounted in 0.1× PBS, 90T glycerol, 0.1% *P*-penylenediamine (pH 8.0) and examined (400× magnification).
27. C. Schindler, K. Shuai, V. Prezioso, J. E. Darnell, unpublished observations.
28. N. C. Reich and L. M. Pfeffer, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8761 (1990); D. S. Kessler and D. E. Levy, *J. Biol. Chem.* **266**, 23471 (1991).
29. Cells to be extracted for antibody precipitation studies were washed in cold PBS twice. Lysis buffer (1 ml) (0.5% NP-40, 240 mM NaCl, 50 mM Tris (pH 8.0), 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM pMSF, 3 μg/ml Aprotinin, 1 mg/ml Pepstatin, 1 mg/ml Leupeptin) was added and mixed, and the extracts were centrifuged for 1 min in a microfuge. The supernatant was incubated with preimmune serum (2.5 μl) and protein A/G agarose (15 μl) (Oncogene Science) for 60 min and then with 3 μl of anti-113, anti-91, or anti-91T

and protein A/G agarose (15 μl) 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.
30. K. Shuai, C. Schindler, J. E. Darnell, unpublished observations.
31. T. Decker, D. J. Lew, Y.-S. E. Cheng, D. E. Levy, J. E. Darnell, Jr., *EMBO J.* **8**, 2009 (1989).
32. T. Decker, D. J. Lew, J. Mirkovitch, J. E. Darnell, Jr., *ibid.* **10**, 927 (1990).
33. K. Shuai, C. Schindler, V. Prezioso, J. E. Darnell, Jr., unpublished observations.
34. G. Uze, G. Lutfalla, I. Gresser, *Cell* **60**, 225 (1990).
35. J. M. Aguet, Z. Dembic, G. Merlin, *ibid.* **55**, 273 (1988).
36. For analysis of phosphoamino acids and phosphopeptides, the appropriate electrophoretically separated proteins (Fig. 3) were cut out from a 7% 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 NH_4CO_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_2O:pyridine:acetic acids, 15:12:10:3).
37. S. I. Walaas and A. C. Nairn, *J. Mol. Neurosci.* **1**, 117 (1989).
38. Supported by grants from the American Cancer Society (MV-424U) and the National Institutes of Health (AI32489). V.R.P. is supported by training grant GM07982-10. We thank T. Suzuki and others in P. Greengard's laboratory for assistance with phosphoprotein analysis.

11 May 1992; accepted 29 June 1992

Activation of Transcription Factors by Interferon-alpha in a Cell-Free System

Michael David and Andrew C. Larner*

The signal transduction mechanisms of interferons (IFNs) remain unclear partly because no effect of IFN has been reproducible in a cell-free system. IFN-α 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-α to its receptor. A system was developed in which IFN-α activated ISGF3 in homogenates of HeLa cells. Subcellular fractionation revealed that incubation of a plasma membrane–enriched fraction with IFN-α 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-α and IFN-β). The transcribed genes contain within their promoters similar sequences, termed interferon stimulated response elements (ISREs), which are necessary and sufficient for IFN-α–induced expression (*1*, *2*). Two IFN-α–induced protein complexes (ISGF2 and ISGF3) and one constitutive protein complex (ISGF1) have been defined by means of electrophoretic mobility-shift gel assays (EMSAs) with ISRE probes (*1*, *3*, *4*). ISGF3, a multisubunit protein complex, appears to be the primary positive regulator of IFN-α–induced gene transcription (*1*, *3*, *4*). The DNA binding component of the complex, ISGF3γ, 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-γ (*5*, *6*), and the constitutive concentration of ISGF3γ in the cell varies from one cell type to another. ISGF3α 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-α binds to cells, and they then interact with ISGF3γ to activate IFN-α–stimulated genes (*1*, *7*).

We prepared homogenates from HeLa cells that had been exposed to IFN-γ for 8 to 12 hours to induce sufficient concentrations of ISGF3γ so that IFN-α activation of ISGF3α could be detected by EMSA. IFN-α–induced ISGF3 formation in HeLa homogenates exposed to IFN-α 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-α. Addition of IFN-γ, 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-(β,γ-methylene)-diphosphonate (AMP-PCP) did not substitute for ATP (*8*). The ISGF3 complex co-migrated with ISGF3 formed in intact cells exposed to IFN-α (*8*).

To obtain more information concerning IFN-α activation of ISGF3, HeLa cell homogenates were fractionated. We took advantage of the fact that ISGF3γ, the DNA-binding component of ISGF3, is not modified in cells treated with IFN-α 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 2500*g*. The remaining cellular material was then centrifuged at 18,000*g*. 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

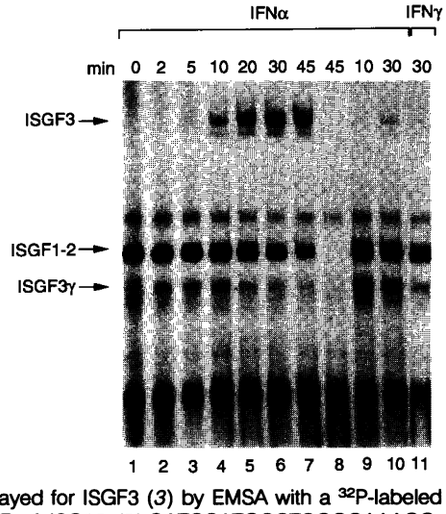

Fig. 1. Activation by IFN-α of formation of ISGF3 in homogenized HeLa cells. HeLa cells (approximately 3 × 10^8) were incubated with recombinant human IFN-γ (1 ng/ml) (Genentech) for 12 hours. Cells were collected by centrifugation, washed with phosphate-buffered saline, and resuspended in 1.5 ml of reaction buffer [20 mM $MgCl_2$, 200 μM $CaCl_2$, 100 mM NaF, 100 mM Hepes (pH 7.9), 200 μM Na-ascorbate, 12 mM phosphoenolpyruvate, 4 mM ATP, 150 μM GTP-γ-S, pyruvate kinase (30 μg/ml), and 600 μM PMSF]. Cells were homogenized and portions (50 μl) of the lysate were incubated without or with recombinant human IFN-α-2a (Hoffmann–La Roche, 2 × 10^4 units) for the indicated times at 30°C. Incubation was terminated by addition of ice-cold stop solution [1 mM $MgCl_2$, 10 mM KCl, 20 mM Hepes (pH 7.0), 20% glycerol, 500 μM dithiothreitol (DTT), 250 μM PMSF, 0.1% NP-40]. The mixture was centrifuged at 18,000*g* and the supernatant was assayed for ISGF3 (*3*) by EMSA with a ^{32}P-labeled oligonucleotide probe corresponding to the ISRE of ISG15 (5′-GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC-3′) and its complement. Lanes 1 to 7, time course of IFN-α–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-α 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-γ (50 ng/ml) for 30 min.

Division of Cytokine Biology, Center for Biologics Evaluation and Research, Bethesda, MD 20892.

*To whom correspondence should be addressed.