of p91. However, the IL-10 immunoprecipitate also contained a tyrosine phosphorylated protein of 91 kD. This protein did not react with an antibody that recognized both p91 and its 84-kD spliced variant (*7*).

^{32}P-labeled phosphoproteins bound to GRR-agarose were visualized by autoradiography and then incubated with *Y. enterocolitica* PTPase (Fig. 4C) in the absence or presence of orthovanadate. *Yersinia* PTPase selectively hydrolyzed the ^{32}P-labeled IL-3– and GM-CSF–induced 80-kD phosphoprotein, whereas incubation of identical samples in the presence of the PTPase and orthovanadate had no effect. A weaker band of 97 kD was also sensitive to PTPase treatment. These results indicated that the proteins that associated with the GRR were tyrosine phosphorylated as a result of treatment with either IL-3 or GM-CSF.

Although it seems likely that IL-3, IL-5, IL-10, or GM-CSF induce the expression of sets of early response genes that are responsible for their biological actions, no well-defined enhancers have been identified in the promoters of cellular genes that are rapidly activated by these cytokines. IL-10 and IFN-γ activated the formation of a GRR binding complex that contained p91. The presence of p91 in these complexes appears to be important for the ultimate expression of the FcγRI gene, because only IFN-γ and IL-10 treatment resulted in increased expression of the RNA. The complexes activated by IL-3, IL-5, and GM-CSF do not contain either p91 or the 113-kD protein of ISGF-3 (*13–15*), but rather a prominent phosphoprotein of 80 kD. It appears that at least by their relative migration in SDS-PAGE, none of these proteins are identical to the 84-kD component of ISGF-3 that lacks the COOH-terminal 39 amino acids of p91 (*15*). The assembly of these phosphoproteins into DNA binding complexes appears to be a general mechanism by which growth factors can modulate gene expression through activation of putative transcription factors by tyrosine phosphorylation.

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Induction by EGF and Interferon-γ of Tyrosine Phosphorylated DNA Binding Proteins in Mouse Liver Nuclei

Susan Ruff-Jamison, Katherine Chen, Stanley Cohen*

Intraperitoneal injection of epidermal growth factor (EGF) into mice resulted in the appearance in liver nuclei of three tyrosine phosphorylated proteins (84, 91, and 92 kilodaltons) within minutes after administration of EGF. Administration of interferon-γ (IFN-γ) resulted in the appearance in liver nuclei of two tyrosine phosphorylated proteins (84 and 91 kilodaltons). The 84- and 91-kilodalton proteins detected after either EGF or IFN-γ administration were identified as the IFN-γ activation factors (GAF). Furthermore, gel shift analysis revealed that these GAF proteins, detected after either EGF or IFN-γ administration, specifically bound to the sis-inducible element of the c-*fos* promoter. Thus, GAF proteins participate in nuclear signaling in both IFN-γ and EGF pathways.

Epidermal growth factor elicits a wide range of physiological responses from a variety of cell types (*1–3*). All of these responses are believed to be mediated by the membrane-spanning receptor for EGF (EGFR). Ligand binding by the EGFR activates its intrinsic tyrosine kinase activity and results in many cellular alterations, including the generation of signals that activate transcription (*4–6*) and the tyrosine phosphorylation of cytoplasmic proteins (*7–10*). We have previously reported that the injection of EGF into mice leads to increased tyrosine phosphorylation of a number of proteins in all organs examined (*11, 12*).

To detect signaling proteins that might be involved in transcriptional regulation, we isolated liver nuclei and examined them for the presence of tyrosine phosphorylated proteins before and after the administration of EGF (Fig. 1A). A tyrosine phosphorylated protein of approximately 92 kD was readily apparent in nuclei 6 and 20 min after the administration of EGF. The extent of tyrosine phosphorylation of the 92-kD protein was diminished by 60 min and was detectable only in trace amounts in nuclei from control mice. The 92-kD protein could not be extracted from nuclei with 1.0% Triton X-100, indicating that the protein was inside the nucleus and not a cytoplasmic contaminant or a protein associated with the outer nuclear membrane (*13*). However, more than 90% of this protein could be extracted with 0.2 M NaCl (Fig. 1B). Because the 0.2 M NaCl extraction procedure partially purified the 92-kD protein from the nucleus, the material extracted in this way was used in all subsequent protein characterizations. EGF also induced the appearance of a cytoplasmic 92-kD tyrosine phosphorylated protein (*13*).

Treatment of responsive cells with interferon-α (IFN-α) induces the formation of a tyrosine phosphorylated complex consisting of three protein subunits (IFN-stimulated gene factor–3α, ISGF-3α) that is translocated into the nucleus and forms, with the addition of a fourth subunit (ISGF-3γ), a DNA binding complex specific for the IFN-stimulated response element (ISRE) present in promoters of IFN-α–responsive genes (*14–18*). Two of the components of the ISGF-3α complex are a 91-kD protein and an 84-kD protein, the latter being identical to the 91-kD species but lacking the COOH-terminal 36 amino acids. Treatment of responsive cells with IFN-γ induces the tyrosine phosphorylation of an IFN-γ activation factor (GAF), a DNA binding protein that binds to a sequence (IFN-γ activation site, GAS) required for the tran-

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232.

*To whom correspondence should be addressed.

scriptional activation of a guanylate binding protein (GBP) gene (*19–21*). Both GAF and ISGF-3α contain the same 91-kD tyrosine phosphorylated subunit, and the 91-kD protein contains a src homology region 2 (SH2) domain, suggesting that it interacts with tyrosine phosphorylated proteins (*22*).

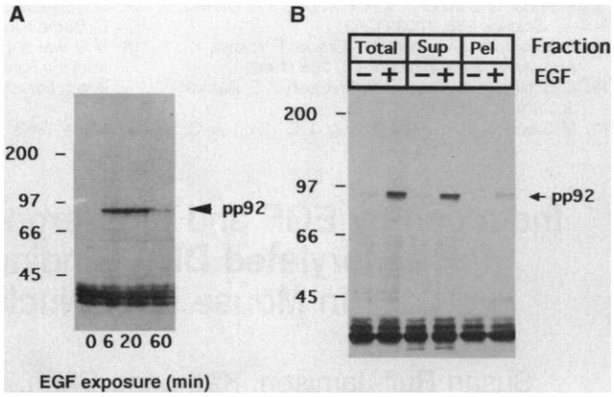

Fig. 1. EGF-induced tyrosine phosphorylation of proteins in liver nuclei. (**A**) Time course. Solutions of EGF (1 mg/ml) or phosphate-buffered saline (PBS) (10 μl per gram of body weight) were injected intraperitoneally into mice (*12*). Mice were killed by cervical dislocation at the indicated times, and liver nuclei were isolated by a modification of a published procedure (*26*). Portions (20 μl) of the nuclear suspension were resolved by SDS-PAGE (7.5%), transferred to Immobilon (Millipore), and blocked with 1% bovine serum albumin in wash buffer [10 mM tris (pH 7.5), 0.1 M NaCl, and 0.1% Tween-20] for 20 min. The Immobilon filter was then immunoblotted with horseradish peroxidase–conjugated recombinant antibody fragment specific for phosphotyrosine, RC20H (diluted 1:2500 in blocking buffer; Transduction Laboratories, Lexington, Kentucky) (*27*), for 20 min and washed in wash buffer. Proteins were detected by enhanced chemiluminescence (ECL, Amersham). (**B**) Salt extraction of the tyrosine phosphorylated proteins from mouse liver nuclei. Nuclei were isolated as described (A). The intact nuclei were centrifuged at 600*g* for 10 min. The sedimented material was extracted with buffer A containing 0.2 M sodium chloride (250 μl per gram of original wet weight) for 30 min on ice with occasional mixing. The nuclear suspension was centrifuged at 15,000*g* for 5 min, and the supernatant was centrifuged at 100,000*g* for 1 hour. Proteins in equivalent portions of total nuclei (20 μl), salt extract, and residual nuclear pellet were resolved by SDS-PAGE (7%), transferred to Immobilon, immunoblotted with RC20H, and detected with ECL. Molecular sizes are indicated at left (in kilodaltons).

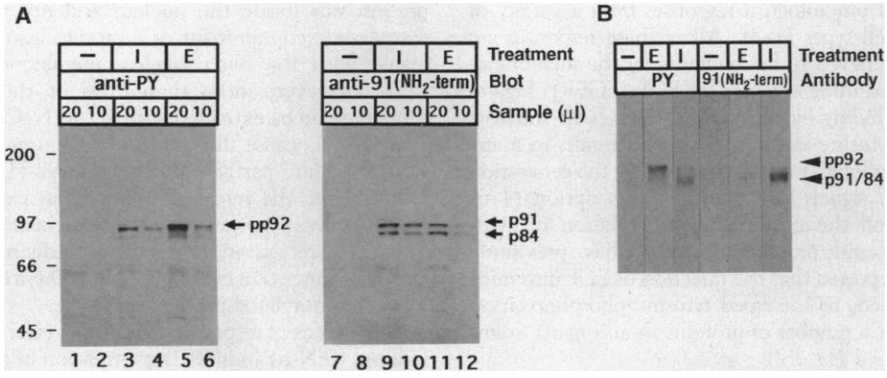

Fig. 2. Pattern of tyrosine phosphorylated and IFN-regulated proteins in liver nuclei after administration of IFN-γ or EGF. Solutions of EGF (1 mg/ml), murine IFN-γ (1 mg/ml), or PBS were injected intraperitoneally into mice, and liver nuclear extracts were prepared as described (Fig. 1). (**A**) Immunoblotting after SDS-PAGE. Portions (20 μl) of nuclear extracts prepared from control (−) or from IFN-γ– (I) or EGF- (E) treated mice were resolved by SDS-PAGE (7%), transferred to Immobilon, immunoblotted with RC20H, and detected with ECL. The blots were then stripped with a solution containing 7 M guanidine hydrochloride, 50 mM glycine (pH 10.8), 0.05 mM EDTA, 0.1 M KCl, and 20 mM mercaptoethanol for 10 min at room temperature with shaking, rinsed thoroughly with water, and immunoblotted with a 1:5000 dilution of anti-91N (Transduction Laboratories, Lexington, Kentucky) for 1 hour. The filters were washed with wash buffer and again immunoblotted with a 1:5000 dilution of goat antibody to rabbit immunoglobulin G labeled with horseradish peroxidase for 1 hour. The blots were washed with wash buffer, and antibody binding was detected with ECL. Molecular sizes are indicated at left (in kilodaltons). (**B**) Immunoblotting after native-PAGE. Portions (5 μl) of nuclear extracts from control (−) or from IFN-γ– (I) or EGF- (E) treated mice were adjusted to 3 mM EDTA, 1 mM dithiothreitol (DTT), and 4% Ficoll in a final volume of 20 μl and were resolved on a native-PAGE (4%) containing 0.5× tris-borate EDTA (TBE) buffer [25 mM tris, 25 mM boric acid, 0.5 mM EDTA). Gels were run at 150 V at room temperature for 2 to 3 hours in 0.5× TBE buffer. Proteins were transferred to Immobilon and immunoblotted first with RC20H and then with anti-91N as described (A).

We tested whether the 92-kD tyrosine phosphorylated protein seen in the liver nuclei in response to EGF was related to the 91-kD tyrosine phosphorylated protein detected in response to IFN. Liver nuclei were isolated from control, EGF-treated, and IFN-γ–treated mice, and 0.2 M NaCl extracts were examined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with antibodies to phosphotyrosine (anti-phosphotyrosine) and antibodies to the NH_2-terminus (anti-91N) or COOH terminus (anti-91C) of the 91-kD protein (Fig. 2A). The 84- and 91-kD tyrosine phosphorylated proteins were present in the extract from IFN-γ–treated mice but not in extracts from control mice. The nuclear extract from EGF-treated mice contained three tyrosine phosphorylated bands (84, 91, and 92 kD). Reprobing of the same blot with anti-91N revealed that the IFN-induced 84- and 91-kD proteins were present in nuclei of both IFN-γ– and EGF-treated mice but not in nuclei from control mice. Reprobing of the blot with anti-91C yielded similar results (*13*). However, neither antibody reacted to the 92-kD tyrosine phosphorylated protein in nuclear extracts from EGF-treated mice (compare lanes 5 and 6 with lanes 11 and 12).

Two experiments were done to more clearly resolve the pattern of tyrosine phosphorylated proteins detected by immunoblot analysis after SDS-PAGE. First, nuclear extracts were resolved by native-PAGE and, after transfer, monitored by immunoblotting with both anti-phosphotyrosine and anti-91N. Major and minor tyrosine phosphorylated proteins were present in extracts from both EGF- and IFN-γ–treated mice but not in extracts from control mice (Fig. 2B). However, a major phosphotyrosine-containing protein was present in the extracts from EGF-treated mice that was not seen in the extracts from IFN-γ–treated mice. Anti-91N reacted to a protein present in extracts from both EGF- and IFN-γ–treated mice but not in extracts from control mice. However, anti-91N did not react to the major EGF-induced tyrosine phosphorylated protein. In a second experiment, proteins in nuclear extracts from control mice or from IFN-γ– or EGF-treated mice were immunoprecipitated with anti-91C, and the precipitated proteins and proteins in the supernatant were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine and anti-91C (Fig. 3). Anti-91N reacts with both the 91- and 84-kD components, whereas anti-91C reacts only with the 91-kD protein. Anti-91C precipitated most of the tyrosine phosphorylated protein in extracts from IFN-γ–treated mice but only some in extracts from EGF-treated mice (Fig. 3A). The supernatants remaining after immunoprecipitation

were free of the 91-kD protein (Fig. 3B). The immunoprecipitates from both IFN-γ– and EGF-treated mice contained the 91-kD protein, but more of the protein was present after IFN-γ treatment (Fig. 3B). The 92-kD tyrosine phosphorylated protein induced by EGF was not immunoprecipitated or immunoblotted by anti-91C (Fig. 3, A and B; compare lanes 6).

We conclude that (i) both the 91- and 84-kD components of the ISGF-3α complex were tyrosine phosphorylated and appeared in the nucleus in response to either IFN-γ or EGF, (ii) the 92-kD phosphotyrosine-containing protein was present in mouse liver nuclei after EGF treatment, and (iii) the IFN proteins were not associated with the 92-kD protein (these proteins did not coimmunoprecipitate with either of two antibodies to the 91-kD protein and they migrated to unique positions on a native gel as determined by immunoblot analysis).

Because EGF and sis platelet-derived growth factor induce the activation of a factor (sis-inducible factor, SIF) that binds to a specific DNA element (sis-inducible element, SIE) in the c-*fos* promoter (*23–25*), gel shift analyses were done with a ^{32}P-labeled SIE (*25*) with nuclear extracts from control or from IFN-γ– or EGF-treated mice. Proteins in nuclear extracts from either IFN-γ– or EGF-treated mice formed specific binding complexes with SIE, although with differing mobility patterns; nuclear extracts from control mice had no binding activity (Fig. 4A). Proteins in nuclear extracts from EGF-treated mice formed three distinct complexes with the SIE, consistent with previous findings (*24*). All of the complexes formed with the SIE in extracts from either IFN-γ– or EGF-treated mice were disrupted by excess unlabeled SIE but not excess ISRE. Excess unlabeled GBP oligonucleotide blocked SIE binding in the IFN-γ–induced complex and components C and B of the EGF-induced complex but did not block binding of component A in the EGF-induced complex. These results suggest that IFN-γ–stimulated response proteins are able to bind SIE and that at least two of the three EGF-induced SIE binding complexes may be related to the IFN-γ–induced binding proteins.

The relatedness of the IFN-γ– and EGF-activated DNA binding proteins was further examined in a supershift experiment carried out with anti-91C. Anti-91C shifted all of the observed protein-DNA complexes in extracts from IFN-γ–treated mice and all of complex C formed with nuclear extracts from EGF-treated mice (Fig. 4B). However, this antibody supershifted only a fraction of complex B and did not appear to alter the mobility of complex A. Anti-91N produced identical results. Control antibodies had no effect on band shift mobility (*13*). These results confirm that the IFN-γ–stimulated response proteins bind to the SIE and that two of the three EGF-induced SIE binding complexes contain the IFN-γ–stimulated response proteins.

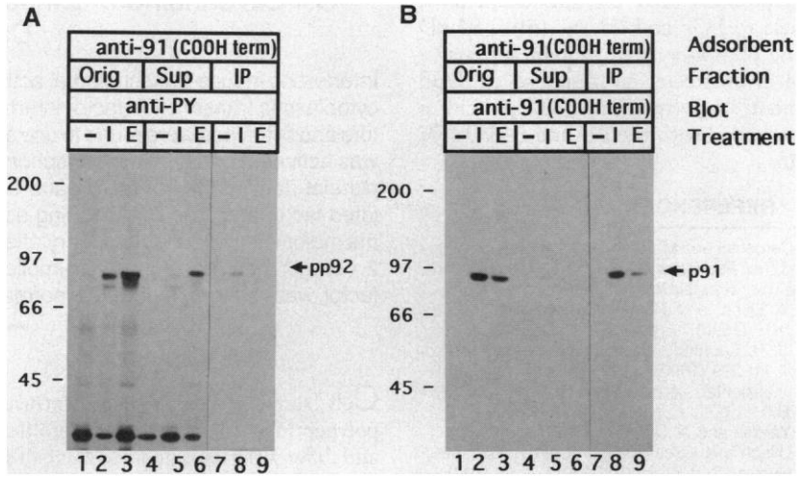

Fig. 3. Immunoprecipitation of proteins from nuclear extracts with anti-91C. Portions (40 μl) of nuclear extracts from control (–) or from IFN-γ– (I) or EGF- (E) treated animals were added to 160 μl of buffer B [10 mM tris (pH 7.5), 0.1 M NaCl, and 3 mM EDTA]. These extracts were immunoprecipitated with anti-91C (2 μg) for 4 hours at 4°C and then incubated for 1 hour with 50 μl of protein A–Sepharose (50% slurry). The suspensions were centrifuged, and the resulting supernatants were retained for immunoblot analysis. The sedimented material was washed three times with buffer B, and the bound proteins were eluted by boiling in 2× Laemmli buffer (100 μl) (*28*) for 5 min. Equivalent portions of original extracts, immunoprecipitation supernatants, and sedimented material were resolved by SDS-PAGE (7%), transferred to Immobilon, and immunoblotted with RC20H (**A**). Blots were stripped and reprobed with anti-91C (**B**) as described (Fig. 2A). Molecular sizes are indicated at left (in kilodaltons).

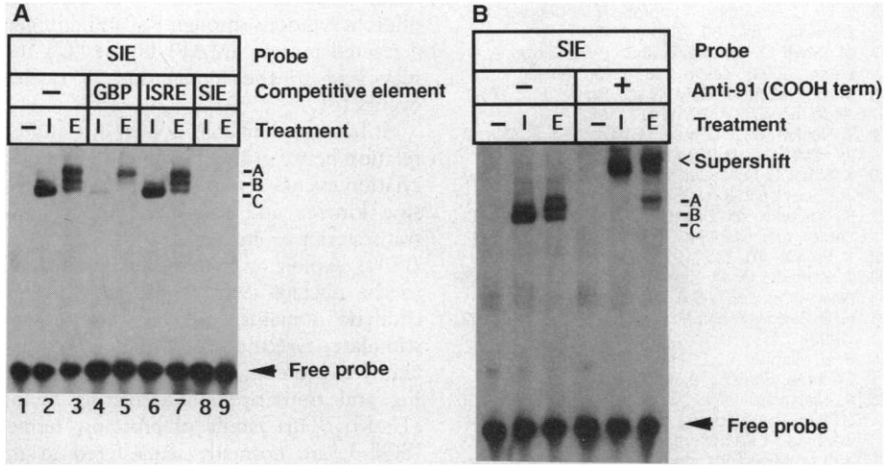

Fig. 4. Mobility-shift assays of SIE binding proteins present in nuclear extracts. Nuclear extracts were prepared from control or from IFN-γ– or EGF-treated mice as described (Fig. 1). The sequences of the elements used in the gel shift analysis were as follows: GBP, 5′-AAGTACTTTCAGTTTCATATTACTCTAAATC-3′; ISRE-15, 5′-GATCGGGAAAGGGAAACCGAAACTGAAGCC-3′; and SIE, 5′-GTGCATTTCCCGTAAATCTTGTCTACAATTC-3′. The oligonucleotides were labeled with [^{32}P]adenosine triphosphate and T4 polynucleotide kinase (BRL). (**A**) Specificity of SIE binding. All incubations were done at room temperature. Nuclear extract (5 to 10 μg) from control (–) or from IFN-γ– (I) or EGF- (E) treated animals were incubated with poly(dI-dC)·poly(dI-dC) (2 μg, Pharmacia) in 3 mM EDTA, 1 mM DTT, and 4% Ficoll in a final volume of 20 μl for 10 min. A second 10-min incubation was done with a 50 times larger concentration of the indicated competitive oligonucleotide. Finally, labeled SIE oligonucleotide (1 × 10^5 cpm) was added for 15 min. The reaction was then resolved on a native-PAGE (4%) containing 0.5× TBE buffer for 2 to 3 hours at 150 V. The gel was then dried and exposed to film. (**B**) Supershift analysis of SIE binding proteins. Gel shift analysis was done as in (A) except that, where indicated, anti-91N (1 μg) was substituted for the competing oligonucleotide in the second 10-min incubation period.

We conclude that (i) EGF induces the tyrosine phosphorylation and nuclear translocation of the 91- and 84-kD IFN–stimulated response proteins, (ii) the 91- and 84-kD proteins bind the SIE element in response to EGF and IFN-γ, (iii) a 92-kD tyrosine phosphorylated protein appears in mouse liver nuclei in response to EGF treatment, and (iv) the 92-kD protein is not associated with the 91- and 84-kD IFN proteins.

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Ras-Independent Growth Factor Signaling by Transcription Factor Tyrosine Phosphorylation

Olli Silvennoinen, Christian Schindler, Joseph Schlessinger, David E. Levy*

Interferons induce transcriptional activation through tyrosine phosphorylation of the latent, cytoplasmic transcription factor interferon-stimulated gene factor–3 (ISGF-3). Growth factors and cytokines were found to use a similar pathway: The 91-kilodalton subunit of ISGF-3 was activated and tyrosine phosphorylated in response to epidermal growth factor (EGF), platelet-derived growth factor, and colony stimulating factor–1. The tyrosine phosphorylated factor acquired DNA binding activity and accumulated in nuclei. Activation required the major sites for autophosphorylation on the EGF receptor that bind Src homology region 2 domain–containing proteins implicated in Ras activation. However, activation of this factor was independent of the normal functioning of Ras.

Cell surface receptors for extracellular polypeptide ligands transduce proliferative and differentiation signals to the cell's interior. Many growth factor receptors are tyrosine kinases, containing a cytoplasmic catalytic domain capable of both autophosphorylation and phosphorylation of cellular substrates after ligand activation (*1*, *2*). Autophosphorylated receptors serve as docking sites for proteins that contain Src homology region 2 (SH2) domains (*3*). One such protein, Grb2 (*4*), serves as a link in the activation of Ras (*5*), a common response for receptor and nonreceptor signaling pathways (*6*). Activated Ras initiates a cascade of serine and threonine phosphorylations through Raf and mitogen-activated protein (MAP) kinases (*7*) that may lead to the modulation of nuclear events (*8*).

It has been difficult to establish a direct relation between the cytoplasmic phosphorylation events stimulated by receptor tyrosine kinases and the induction of gene transcription. In contrast, interferons (IFNs) exploit an apparently simpler path to the nucleus (*9*). Despite their lack of catalytic domains (*10*), IFN-α receptors stimulate tyrosine phosphorylation of a family of proteins that serve as DNA binding and transcriptional-activating factors (*11–13*). This family of proteins, termed ISGF-3, are normally sequestered in the cytoplasm (*14*, *15*). After tyrosine phosphorylation they assemble into a multimeric complex, translocate to the nucleus, and bind cis-acting enhancer elements in the regulatory regions of IFN-stimulated genes. IFN-γ stimulates phosphorylation of the 91-kD subunit of ISGF-3 (p91), which activates transcription through a distinct genetic element linked to IFN-γ–responsive genes (*12*). We have found that several polypeptide growth factors that signal through receptor tyrosine kinases also stimulate tyrosine phosphorylation and nuclear accumulation of p91. These findings demonstrate a direct link between growth factor receptor tyrosine kinases and gene transcription.

A nuclear factor, termed sis-inducible factor (SIF), binds a regulatory element in the c-*fos* promoter [sis-inducible element (SIE)] and is activated in response to platelet-derived growth factor (PDGF) (*16*) in a manner reminiscent of ISGF-3 activation (*17*). We treated Swiss 3T3 cells with PDGF for 15 min and tested nuclear and cytoplasmic extracts by electrophoretic band shift assay for the ability to bind to a SIF recognition sequence (*18*). Little or no specific DNA binding activity was present in extracts from untreated cells, but abundant activity was detected in extracts from PDGF-stimulated cells (Fig. 1A). We detected DNA binding activity in both cytoplasmic and nuclear fractions, suggesting that activation may occur in the cytoplasm in a manner similar to ISGF-3 activation (*15*). The DNA binding activity of SIF can be stimulated in vitro in cytoplasmic extracts supplemented with membranes, further supporting the evidence that factor activation occurs in the cytoplasm (*19*).

This DNA binding activity of SIF was detected in extracts from normal human fibroblasts treated with epidermal growth factor (EGF) (Fig. 1B) but not in untreated cells or in cells treated with fibroblast growth factor (FGF). SIF band shift activity was also detected in extracts of cells treated with either IFN-α or IFN-γ. Activation by PDGF, IFN-α, and IFN-γ was rapid, transient, and resistant to protein synthesis inhibition by cycloheximide (Fig. 1C). The duration of the responses differed: SIF ac-

O. Silvennoinen and J. Schlessinger, Department of Pharmacology, New York University School of Medicine, New York, NY 10016.
C. Schindler, Columbia University College of Physicians and Surgeons, New York, NY 10032.
D. E. Levy, Department of Pathology and Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, NY 10016.

*To whom correspondence should be addressed.