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25. Human FS2 fibroblasts were labeled with [^{32}P]orthophosphate (0.8 mCi/ml) for 2 hours and either left unstimulated or stimulated with IFN-α, IFN-γ, or PDGF, as indicated. Lysates were subjected to immunoprecipitation with anti-p91 (*11*) and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), phosphoamino acid analysis, and phosphopeptide analysis, as described (*37*).
26. Cells were lysed in 50 mM tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 500 μM sodium orthovanadate, 1 mM EDTA, 1 mM phenylmethylsuflonyl fluoride, aprotinin (3 μg/ml), and leupeptin (0.5 μg/ml) and immunoprecipitated with anti-p91. Immune complexes were separated on SDS-PAGE and immunoblotted with monoclonal antibodies to phosphotyrosine (Upstate Biotechnology, ICN, and Oncogene Science) or anti-p91 and horseradish peroxidase–labeled second antibody and detected by enhanced chemiluminescence (Amersham).
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39. We thank N. Li for cell lines expressing mutant EGFR, E. Skolnik for cell lines expressing dominant negative Ras, M. Mohammadi for WT7 cells, M. Roussel for cell lines expressing CSF-1 receptor, and W. Li for advice on experimental procedures and for helpful discussions. Supported by grants from NIH (AI-28900), the American Cancer Society, and the Cancer Research Institute (D.E.L.) and from Sugen, Inc. (J.S.). Computing services were supported by NSF. D.E.L. is a Pew Scholar in the Biomedical Sciences.

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A Common Nuclear Signal Transduction Pathway Activated by Growth Factor and Cytokine Receptors

Henry B. Sadowski, Ke Shuai, James E. Darnell Jr., Michael Z. Gilman*

Growth factors and cytokines act through cell surface receptors with different biochemical properties. Yet each type of receptor can elicit similar as well as distinct biological responses in target cells, suggesting that distinct classes of receptors activate common gene sets. Epidermal growth factor, interferon-γ, and interleukin-6 all activated, through direct tyrosine phosphorylation, latent cytoplasmic transcription factors that recognized similar DNA elements. However, different ligands activated different patterns of factors with distinct DNA-binding specificities in the same and in different cells . Thus, unrelated receptors may activate a common nuclear signal transduction pathway that, through differential use of latent cytoplasmic proteins, permits these receptors to regulate both common and unique sets of genes.

Growth factor receptors have large cytoplasmic domains endowed with protein tyrosine kinase activity (*1*), whereas cytokine receptors generally lack identifiable catalytic activity (*2*). Nevertheless, both classes of receptor can activate both overlapping and distinct sets of genes and biological responses in target cells (*2*, *3*). The c-*fos* protooncogene is activated by both classes of receptor (*2*, *3*). Activation of c-*fos* transcription involves the combined action of several transcription factors, including serum response factor (SRF), Elk-1, and sis-inducible factor (SIF) (*4*). Certain growth factor signals activate c-*fos* transcription through a complex of SRF and Elk-1 (*5*, *6*), whereas other signals require SRF but not Elk-1 (*6*, *7*). Some of these signals may act through SIF, a DNA-binding activity that is rapidly induced by treatment of cells with polypeptide growth factors (*8*–*10*). SIF binds to an element termed the sis-inducible element (SIE) located 25 base pairs (bp) upstream of the c-*fos* SRE (*8*), and an SIE is sufficient under certain circumstances to mediate growth factor–activated transcription (*9*).

Gene activation by interferon-α (IFN-α) is dependent on regulatory sequences termed interferon (IFN)-stimulated response elements (ISREs) and on the transcription factor IFN-stimulated gene factor–3 (ISGF-3) (*11*). ISGF-3 consists of three polypeptides of 113, 91 or 84, and 48 kD (*12*, *13*). Treatment of cells with IFN-α results in the migration of the 113- and 91-kD proteins to the nucleus, where they interact with the 48-kD subunit to constitute ISGF-3 DNA-binding activity (*13*). Activation of ISGF-3 is associated with phosphorylation of the 113- and 91-kD subunits on tyrosine residues, suggesting that their activity may be directly regulated by tyrosine phosphorylation (*14*). IFN-γ acts through a similar mechanism, although the factor responsible, IFN-γ activation factor (GAF), consists solely of the 91-kD subunit of ISGF-3 (*15*). The 113- and 91-kD proteins, which are related in structure (*16*, *17*), have been termed signal transducers and activators of transcription, or STATs (*18*). Here we show that some forms of the growth factor–activated SIF contain the Stat91 protein.

To identify the polypeptides that constitute SIF activity, we performed DNA-affinity precipitation assays (*19*). A431 human epidermoid carcinoma cells were metabolically labeled with [^{35}S]methionine, and half of the cells were treated with epidermal growth factor (EGF) to induce SIF activity. Extracts prepared from the cells were incubated with streptavidin-conjugated paramagnetic beads bound to a biotinylated oligonucleotide carrying a variant SIF binding site, m67 (Table 1), that binds SIF with higher affinity than the human c-*fos* SIE (*9*). The beads were washed extensively, and tightly bound proteins were eluted with 2 M NaCl. Several polypeptides from EGF-treated cells bound to the m67 oligonucleotide (Fig. 1). Four of these polypeptides (80 to 90 kD) were not detected in extracts of untreated cells. These proteins were not

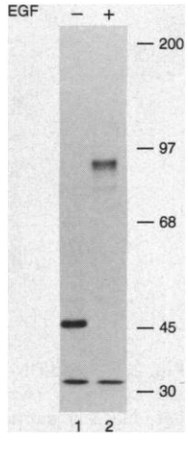

Fig. 1. DNA-affinity precipitation analysis of SIE-binding proteins from untreated and EGF-treated A431 cells. A431 cells were labeled for 3 hours with [^{35}S]methionine (200 μCi/ml) and treated with either PBS (lane 1) or recombinant human EGF (100 ng/ml, lane 2) for 15 min. Cytoplasmic extracts were prepared, and ^{35}S-labeled SIE-binding proteins were collected on ligated high-affinity SIE oligonucleotide bound to streptavidin-conjugated paramagnetic beads. After washing, retained polypeptides were eluted with 2 M NaCl, separated by SDS-PAGE, and visualized by fluorography. Molecular size standards are indicated at right (in kilodaltons).

H. B. Sadowski and M. Z. Gilman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.
K. Shuai and J. E. Darnell Jr., Laboratory of Molecular Cell Biology, Rockefeller University, New York, NY 10021.

*To whom correspondence should be addressed.

bound by an oligonucleotide carrying a mutant SIE, m34 (*9*, *20*). Thus, these proteins are candidate subunits of EGF-induced SIF activity in A431 cells. Some of these proteins are similar in size to Stat91.

SIF activity, as measured in a mobility-shift assay using the m67 sequence, was not competed by a large molar excess of an oligonucleotide carrying an ISGF-3 binding site (*20*, *21*), indicating that SIF and ISGF-3 have nonoverlapping DNA-binding specificities. In contrast, binding sites for SIF and GAF did compete with one another. SIF activity induced by EGF in A431 cells consists of three complexes (*10*), A, B, and C (Fig. 2A) (*22*). Binding of all three SIF complexes was inhibited by a molar excess of unlabeled oligonucleotide carrying the high-affinity SIE. SIF binding was also reduced by an oligonucleotide carrying the IFN-γ activation site (GAS) from the Ly-6E gene (*23*), which binds GAF. The GAS oligonucleotide was generally a poorer competitor than the SIE, but it preferentially inhibited binding of SIF-C. These data indicate that SIF can bind a GAS sequence. Furthermore, they suggest that the different SIF complexes have distinct DNA-binding specificities: the GAS sequence binds SIF-C with greater affinity than SIF-A and SIF-B.

Table 1. Representative binding sites for STAT factors. Shown are sequences discussed in the text. Underlined sequences indicate a short region of partial dyad symmetry (TTCCNGTAA) shared by these sites. The APRE is shown in reverse orientation. Ref., reference.

Site	Sequence	Ref.
Human c-*fos* SIE	CAGTTCCCGTCAATC	(*8*)
High-affinity SIE (m67)	CATTTCCCGTAAATC	(*9*)
Ly-6E GAS	ATATTCCTGTAAGTG	(*23*)
Human α^2-macroglobulin APRE	CCATTCCCGTAAGAG	(*24*)

Treatment of FS2 cells with IFN-γ resulted in the induction of GAF, as detected by a GAS probe (Fig. 2A). This complex comigrated with SIF-C and was competed both by a homologous GAS oligonucleotide and by the high-affinity SIE oligonucleotide. As observed in the SIF assay, the SIE was a more effective competitor than the GAS sequence. Taken together, these data show that SIF and GAF have related, though not identical, DNA-binding specificities.

We next asked whether EGF treatment induced GAF and IFN-γ induced SIF. EGF treatment of A431 cells resulted in the appearance of a GAS-binding activity that comigrated with authentic GAF from IFN-γ–treated FS2 cells (Fig. 2B). The strong band that migrates above GAF in the A431 samples is a complex specific to these cells that is not ligand-inducible and does not bind the SIE (*20*); we have not characterized it further. Reciprocally, treatment of FS2 cells with IFN-γ resulted in the appearance of an activity that bound to the human c-*fos* SIE and comigrated with SIF-C. Thus, EGF induces a GAF-like activity and IFN-γ induces a SIF-like activity.

There are differences in the activities induced by the two ligands. On the human c-*fos* SIE, IFN-γ induces exclusively SIF-C, whereas EGF induces all three SIF complexes, with SIF-A being most prominent. This difference is not due to differences in the cells because, whereas treatment of A431 cells with EGF induced formation of all three SIF complexes on the high-affinity SIE, IFN-γ treatment of the same cells induced primarily SIF-C and some SIF-B.

We also assayed extracts from HepG2 human hepatoma cells treated with interleukin-6 (IL-6), a major mediator of the acute phase response in liver. IL-6 induces a DNA-binding activity that acts through an acute phase response element (APRE) related in sequence to SIE and GAS (Table 1) (*24*). Like EGF and IFN-γ, IL-6 also induced activities that bound to the m67 SIE (Fig. 2C). At low IL-6 concentrations, SIF-A was the predominant complex induced, whereas at higher IL-6 concentrations, SIF-B and SIF-C were also formed. In

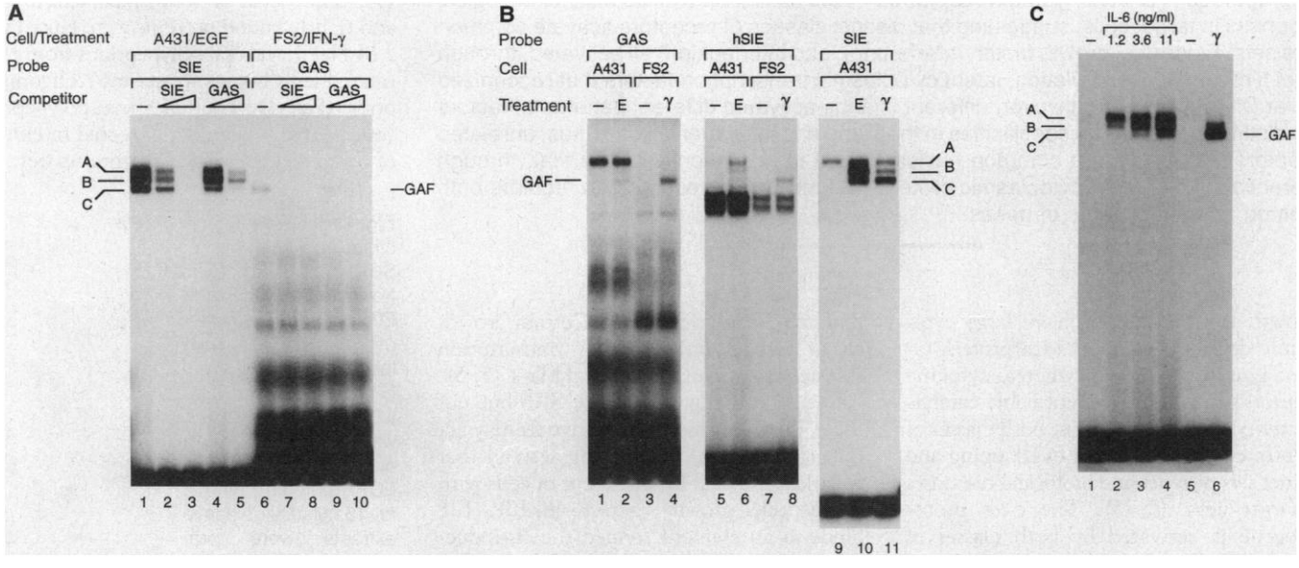

Fig. 2. Related DNA-binding activities induced by different growth factors and cytokines. (**A**) Competition analysis of SIE- and GAS-binding activities. Nuclear extract from EGF-treated (15 min, 100 ng/ml) A431 cells lanes were subjected to mobility-shift assay using a high-affinity SIE probe alone (lane 1) or in the presence of 5- and 50-fold molar excess of unlabeled SIE (lanes 2 and 3) or GAS oligonucleotides (lanes 4 and 5). Whole-cell extracts from FS2 cells treated for 15 min with recombinant human IFN-γ (5 ng/ml) were assayed with ^{32}P-labeled GAS probe alone (lane 6) or in the presence of 5- and 50-fold molar excess of unlabeled SIE (lanes 7 and 8) or GAS oligonucleotides (lanes 9 and 10). (**B**) Mobility-shift assays with GAS (lanes 1 to 4), human c-*fos* SIE (lanes 5 to 8), and high-affinity SIE (lanes 9 to 11) probes. Extracts were prepared from untreated A431 cells (lanes 1, 5, and 9), EGF-treated (15 min, 100 ng/ml) A431 cells (lanes 2, 6, and 10), untreated FS2 cells (lanes 3 and 7), IFN-γ–treated (15 min, 5 ng/ml) FS2 cells (lanes 4, 8), or IFN-γ–treated (15 min, 5 ng/ml) A431 cells (lane 11). The strong band migrating above GAF in lanes 1 and 2 is specific to A431 cells. (**C**) Mobility-shift assay of HepG2 cell extracts on high-affinity SIE probe. HepG2 cells were untreated (lanes 1 and 5) or treated for 15 min with the indicated concentrations of IL-6 (lanes 2 to 4) or with IFN-γ (10 ng/ml, lane 6).

these same cells, IFN-γ induced primarily SIF-C, emphasizing again the potential for differential activation of these factors. We conclude that multiple growth factors and cytokines activate transcription factors with related but not identical DNA-binding specificities.

We tested whether SIF activity was affected by antibody to the Stat91 protein that constitutes GAF activity (*25*). Two different antisera to Stat91 (anti-Stat91) inhibited SIF-C and, to a lesser extent, SIF-B from EGF-treated A431 cells and resulted in supershifting of some material (Fig. 3A). The antisera to Stat91 had no effect on SIF-A, even at the highest concentrations used. These data suggest that SIF-B and SIF-C contain Stat91 or an antigenically related protein, but that SIF-A does not.

We have described a cell-free assay for activation of SIF (*10*). This assay allowed us to examine the effect of depleting an in vitro reaction mixture of all material reactive with anti-Stat91 before activation with EGF (*26*). Depletion of extracts with anti-Stat91, but not normal rabbit serum, eliminated the formation of SIF-B and SIF-C (Fig. 3B). Thus, Stat91 (or an antigenically related protein) is required for the formation of two of the SIF complexes.

Because SIF-C behaves identically to GAF in all of our experiments, it is likely that these two proteins are identical. SIF-B may consist of a differentially modified form of Stat91 or a complex of Stat91 with an additional polypeptide. To rule out that SIF-B is a distinct protein antigenically related to Stat91, we prepared cytoplasmic extracts from a cell line, U3, that lacks Stat91 mRNA and protein (*27*). Extracts from U3 cells and its parental cell line 2fTGH were mixed with membrane fractions from A431 cells and subjected to EGF activation in vitro (*26*). Under these conditions, latent SIF activity is supplied by cytosolic fractions (*10*). Cytosol from wild-type 2fTGH cells supported production of all three SIF complexes (Fig. 3C). In contrast, cytosol from U3 cells did not support production of SIF-B and SIF-C. SIF-A was induced normally in U3 cytosol. U3 cells were reconstituted with Stat91 protein by stable transfection of an expression vector containing the Stat91 cDNA (*18*). Cytosol from these reconstituted cells, C91, regained the ability to generate SIF-B and SIF-C in the in vitro assay (Fig. 3C, lanes 5 and 6). Thus, expression of the Stat91 gene is required for formation of SIF-B and SIF-C but not SIF-A. These genetic data suggest that SIF-B and SIF-C contain authentic Stat91.

If some forms of the EGF-activated SIF protein contain Stat91, then one may expect to observe posttranslational modification and nuclear localization of Stat91 in response to EGF treatment (*16*). SIF activity was detectable in cytosolic fractions of EGF-treated A431 cells within 30 s, even when treated at 0°C to slow activation (Fig. 4A). As reported previously, cytosolic SIF was comprised primarily of complexes B and C (*10*). All three complexes appeared in nuclear fractions within 1 min. Protein immunoblots of the same extracts were probed with anti-Stat91 (Fig. 4B) (*28*).

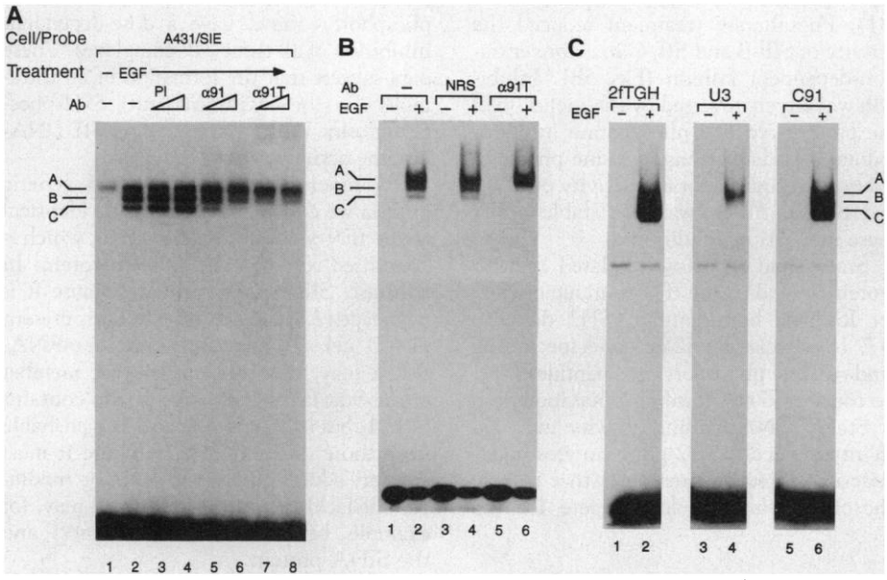

Fig. 3. Evidence that Stat91 is present in SIF complexes. (**A**) Inhibition of SIF complexes B and C with anti-Stat91. Nuclear extracts from untreated (lane 1) or EGF-treated (lanes 2 to 8) A431 cells were incubated for 20 min on ice with 1 μl of PBS (lanes 1 and 2) or 1 μl of the following rabbit antisera diluted 1:10 or 1:3.33 with PBS: preimmune (lanes 3 and 4), anti-91 (lanes 5 and 6), or anti-91T (lanes 7 and 8) (*15–17*). High-affinity SIE probe was added and mobility-shift assays performed. (**B**) Depletion of in vitro activation assay with anti-Stat91. HeLa cell cytosol was incubated overnight at 4°C with PBS (lanes 1 and 2), normal rabbit serum (lanes 3 and 4), or anti-91T (lanes 5 and 6). Immune complexes were collected on protein G–Sepharose beads. Depleted supernatants were mixed with detergent-solubilized A431 membranes and subjected to an in vitro SIF activation assay (*10*). (**C**) In vitro activation assay with cytosol from cell lines containing or lacking Stat91. Portions of cytosol from the indicated cell lines were mixed with detergent-solubilized A431 membranes and subjected to an in vitro activation assay. The 2fTGH cells (lanes 1 and 2) are the parental cell line expressing Stat91. The U3 cells (lanes 3 and 4) are a genetic variant that does not express Stat91 (*27*). The C91 (lanes 5 and 6) cells are a U3-derived line stably transfected with a Stat91 expression vector.

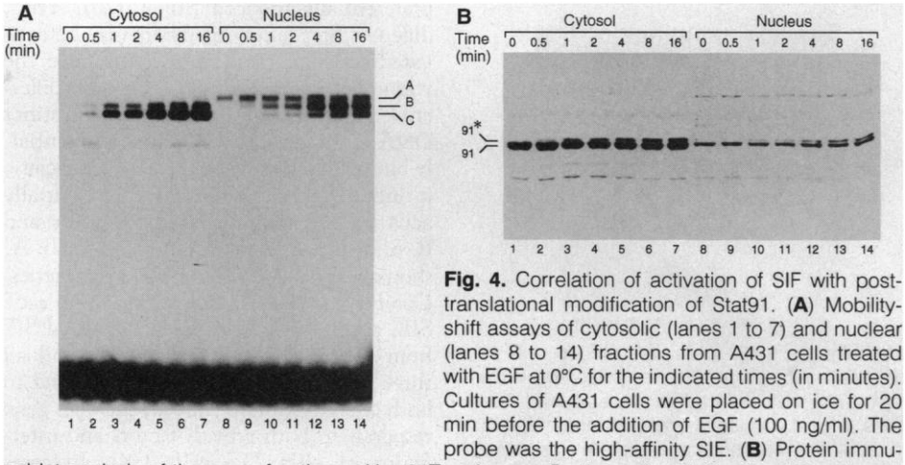

Fig. 4. Correlation of activation of SIF with posttranslational modification of Stat91. (**A**) Mobility-shift assays of cytosolic (lanes 1 to 7) and nuclear (lanes 8 to 14) fractions from A431 cells treated with EGF at 0°C for the indicated times (in minutes). Cultures of A431 cells were placed on ice for 20 min before the addition of EGF (100 ng/ml). The probe was the high-affinity SIE. (**B**) Protein immunoblot analysis of the same fractions with α91T antiserum. Portions of each fraction (30 μg) were denatured by boiling in Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and probed with α91T rabbit polyclonal antiserum as described (*29*). "91" indicates the position of Stat91, and "91*" indicates the more slowly migrating posttranslationally modified form of Stat91 (*16*).

Treatment of A431 cells resulted in the rapid appearance of a lower mobility form of Stat91 characteristic of activation of GAF by IFN-γ (*16*). This form of Stat91, labeled 91*, appeared first in the cytosol and later in nuclear fractions, and its appearance correlated with the detection of SIF activity in these fractions. Thus, modification and nuclear localization of Stat91 is induced by EGF treatment of A431 cells. A similar modification of Stat91 was observed in extracts incubated in vitro with EGF (*20*).

Activation of ISGF-3 by IFN-α and of GAF by IFN-γ is accompanied by phosphorylation of Stat91 on a single tyrosine residue, Tyr^{701}. This tyrosine residue is required for IFN-γ–induced Stat91 activity (*18*). The kinetics of phosphorylation coincide with the shift in Stat91 mobility in SDS gels and the activation of ISGF-3 and GAF DNA-binding activity. To determine whether SIF activation by EGF is also accompanied by tyrosine phosphorylation of its polypeptide components, we incubated nuclear fractions from EGF-treated HER14 cells (*29*) with a monoclonal antibody to phosphotyrosine (*30*). We observed a dose-dependent inhibition of all three isoforms of SIF by the antibody to phosphotyrosine (Fig. 5A), suggesting that all forms of SIF contain phosphotyrosine.

We incubated nuclear extracts with highly purified protein tyrosine phosphatase (*31*). Phosphatase treatment reduced the activity of SIF-B and SIF-C in a concentration-dependent fashion (Fig. 5B). Inhibition was largely reversed by the inclusion of the protein tyrosine phosphatase inhibitor sodium vanadate. Thus, tyrosine phosphorylation is required for the activity of SIF-B and SIF-C. SIF-A was not stable under these incubation conditions.

Stat91 and the closely related Stat113 protein, found in the IFN-α–inducible factor ISGF-3, both contain SH2 domains (*17, 18*). Because SH2 domains specifically bind tyrosine phosphorylated peptides (*32*), we considered the possibility that induction of Stat91 DNA-binding activity involved an intramolecular SH2-phosphotyrosine interaction. If so, we reasoned that soluble phosphotyrosine should compete for the SH2 domain, resulting in inhibition of DNA-binding activity. To test this hypothesis, we incubated extracts containing SIF activity with increasing amounts of either free phosphotyrosine, phosphoserine, or phosphothreonine (Fig. 5C). We found that phosphotyrosine, but not phosphoserine or phosphothreonine, gave a dose-dependent inhibition of all three SIF complexes. These data suggest that the formation of an intramolecular (or an intersubunit) SH2-phosphotyrosine bridge is required for SIF DNA-binding activity.

By functional, antigenic, and genetic criteria we conclude that SIF-C is identical to the IFN-γ–induced factor GAF, which is comprised solely of the Stat91 protein. In contrast, SIF-A lacks Stat91 because it is not reactive with anti-Stat91 and is present in U3 cells, which lack Stat91 mRNA. SIF-A may, however, be another member of the Stat family (*33*). SIF-B also contains Stat91, but its properties are distinguishable from those of GAF, and therefore it must contain additional components or modifications lacking from SIF-C. SIF-B may, for example, be a heterodimer of Stat91 and the SIF-A protein.

Our data suggest that growth factors and cytokines activate a common signal transduction pathway that results in the direct tyrosine phosphorylation of a family of latent cytoplasmic transcription factors. Activation of a common signal transduction pathway could account for the ability of these factors to elicit common responses from their target cells in certain instances. Yet different signaling molecules can have distinct biological activities as well. We have observed at least two potential sources of specificity in our experiments. First, different ligands preferentially induced different SIF complexes. IFN-γ induced primarily SIF-C, whereas IL-6 and growth factors preferentially induced SIF-A (*20*). Thus, different Stat family members may be activated to different degrees to produce the various SIF complexes. Second, the different activated complexes have distinct DNA-binding specificities and preferentially bind different sites. Thus IFN-γ, because it induces primarily SIF-C, preferentially activates GAS sites. Growth factors and IL-6, which preferentially induce SIF-A, should activate a distinct set of genes. Composite sites like the high-affinity m67 SIE, and to a lesser extent the natural SIE from the human c-*fos* promoter, bind all three forms of SIF and should respond to both types of stimuli. Indeed, the c-*fos* gene responds to both growth factors and interferons in NIH 3T3 cells (*34*). In cases where all three SIF complexes are induced, for example, in the presence of high concentrations of ligand or receptor, all three classes of sites should respond.

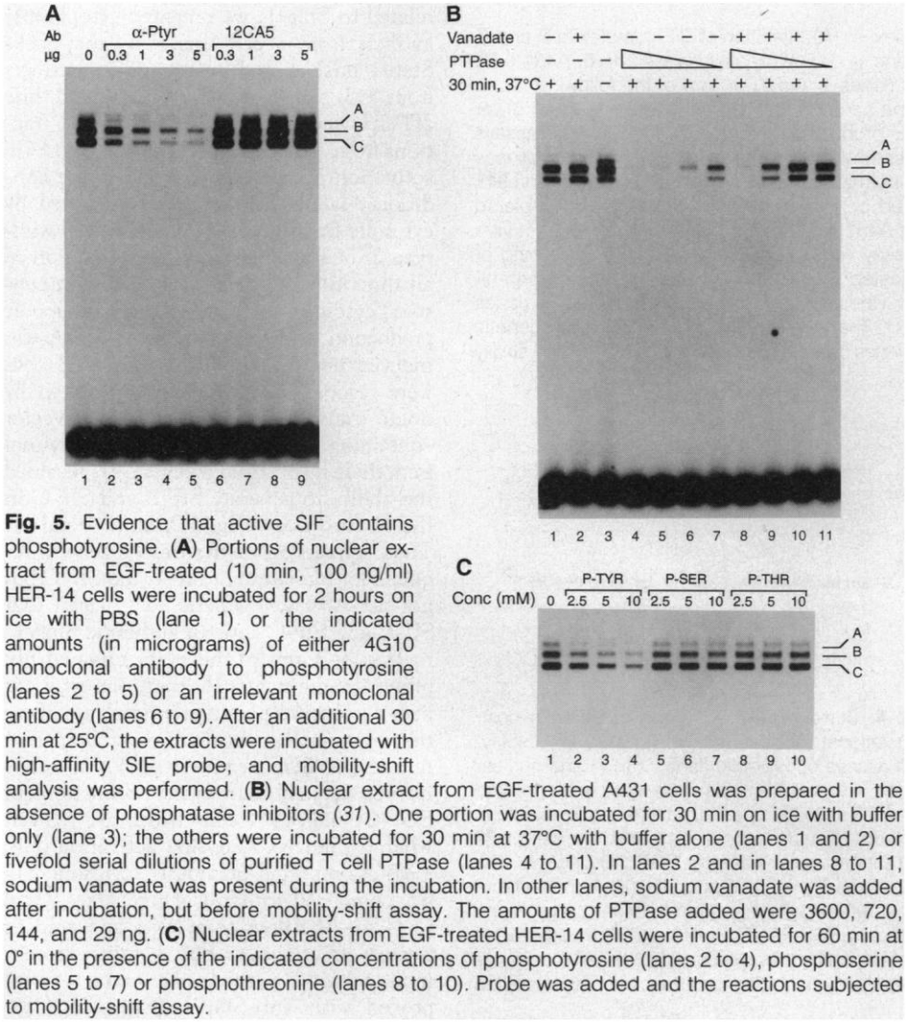

Fig. 5. Evidence that active SIF contains phosphotyrosine. (**A**) Portions of nuclear extract from EGF-treated (10 min, 100 ng/ml) HER-14 cells were incubated for 2 hours on ice with PBS (lane 1) or the indicated amounts (in micrograms) of either 4G10 monoclonal antibody to phosphotyrosine (lanes 2 to 5) or an irrelevant monoclonal antibody (lanes 6 to 9). After an additional 30 min at 25°C, the extracts were incubated with high-affinity SIE probe, and mobility-shift analysis was performed. (**B**) Nuclear extract from EGF-treated A431 cells was prepared in the absence of phosphatase inhibitors (*31*). One portion was incubated for 30 min on ice with buffer only (lane 3); the others were incubated for 30 min at 37°C with buffer alone (lanes 1 and 2) or fivefold serial dilutions of purified T cell PTPase (lanes 4 to 11). In lanes 2 and in lanes 8 to 11, sodium vanadate was present during the incubation. In other lanes, sodium vanadate was added after incubation, but before mobility-shift assay. The amounts of PTPase added were 3600, 720, 144, and 29 ng. (**C**) Nuclear extracts from EGF-treated HER-14 cells were incubated for 60 min at 0° in the presence of the indicated concentrations of phosphotyrosine (lanes 2 to 4), phosphoserine (lanes 5 to 7) or phosphothreonine (lanes 8 to 10). Probe was added and the reactions subjected to mobility-shift assay.

Specificity at the level of subunit activation may result from the differential activation of the tyrosine kinases that regulate DNA-binding activity. These signaling pathways appear to involve a family of large nonreceptor tyrosine kinases that include Tyk2, Jak1, and Jak2 (35). Different members of this kinase family may selectively target unique Stat proteins.

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19. Although cytosol from EGF-treated A431 cells only forms two of the three SIE-binding complexes in mobility-shift assays (*10*), it is relatively free of other DNA-binding proteins, facilitating detection of labeled SIE-binding proteins in a single round of DNA-affinity precipitation. Confluent 15-cm dishes of A431 cells were rinsed twice with prewarmed methionine-free Dulbecco's modified Eagle's medium (DMEM) and labeled in this medium containing 0.5% fetal calf serum (FCS) and [^{35}S]methionine (200 μCi/ml). After 3 hours, phosphate-buffered saline (PBS) or recombinant human EGF (100 ng/ml) was added, and incubation was continued for 15 min. After treatment, the cultures were placed on ice, rinsed with ice-cold DMEM and ice-cold PBS, and the cells were scraped from the dishes in PBS (3 ml) containing 1 mM Na_3VO_4 and 5 mM NaF. The cells were sedimented and resuspended in three packed cell volumes of hypotonic buffer (*10*), swollen for 10 min, and lysed by repeated passage through a 25-gauge needle. Crude nuclei and unbroken cells were sedimented by brief centrifugation (20 s at 16,000*g*). The supernatant was supplemented with NaCl to 120 mM and then clarified by centrifugation (20 min at 16,000*g*), after which glycerol was added to 10% (clarified cytosol). Portions of extracts from control and EGF-treated cells (10^7 cpm) were brought to 0.2 ml with cytosol dilution buffer (CDB, hypotonic buffer with 120 mM NaCl and 10% glycerol) and were supplemented with calf serum to 2.5%, Triton X-100 to 0.05%, and nonspecific competitor [poly(dI-dC) (75 μg/ml) and salmon sperm DNA (15 μg/ml)]. Extracts were then incubated for 1 hour at 4°C with 40 μg of paramagnetic beads (Dynal) containing 24 pmol of high-affinity SIF binding site oligonucleotide [M67; Table 1 and (*9*)]. After 1 hour the beads were separated from the supernatants with a magnetic tube rack, and the beads were washed twice with 0.5 ml of CDB with 0.05% Triton X-100. Proteins were eluted with 20-μl portions of 1 and 2 M NaCl in CDB, denatured by boiling in Laemmli sample buffer, and separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%). For preparation of the affinity matrix, complementary oligonucleotides generating either high-affinity SIF binding site m67 or a mutant binding site m34 (*9*), with 5′ overhangs, were phosphorylated, annealed, and ligated into large arrays. After phenol-chloroform extraction and ethanol precipitation, 10-μg portions (600 pmol of binding site) were biotinylated by incorporation of biotin-16–deoxyuridine triphosphate (biotin-dUTP; Boehringer Mannheim) with the Klenow fragment. After phenol-chloroform extraction, free biotin-dUTP was removed by successive ammonium acetate–ethanol precipitations. DNA-bound streptavidin-conjugated paramagnetic beads were prepared as suggested by the manufacturer, with a coupling efficiency of 10 ng of DNA (600 fmol of binding site) per microgram of beads.
20. H. B. Sadowski and M. Z. Gilman, unpublished results.
21. The ISGF-3 binding site was derived from the ISG15 gene [N. Reich *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6394 (1987)]. The oligonucleotide was provided by N. Reich, State University of New York, Stony Brook.
22. Large-scale nuclear extracts from EGF-treated A431 cells were prepared from hypotonically lysed cells as described (*10*). In experiments with HER14 cells, HepG2 cells or, where multiple treatments of A431 cells were performed, cell lysis and cytosol preparation were as described (*19*). Nuclear extracts were prepared by resuspension of the crude nuclei in 2.5 packed cell volumes of high-salt buffer (hypotonic buffer with 420 mM NaCl and 20% glycerol) and extraction of proteins by rocking for 30 min at 4°C. After centrifugation at 4°C (20 min, 16,000*g*), the supernatants were frozen until use. Whole cell extracts from FS2 cells were prepared as described (*16*). Unless otherwise noted, binding reactions, with in vivo– or in vitro–activated extracts (10 to 20 μg of protein) and labeled oligonucleotide probes (20,000 cpm), and gel mobility–shift assays were performed as described (*10*). High-affinity SIE m67 (*9*) was prepared by annealing the oligonucleotides 5′-GTCGACATTTCCCGTAAATC-3′ and 5′-TCGACGATTTACGGGAAAGT-3′, the human c-*fos* hSIE site by annealing 5′-GTCGACAGTTCCCGTCAATC-3′ and 5′-TCGACGATTGACGGGAACTG-3′, and the Ly-6E GAS site (*23*) by annealing 5′-CATGTTATGCATATTCCTGTAAGTG-3′ and 5′-CATGCACTTACAGGAATATGCATAA-3′. Labeled probes were prepared by Klenow reaction with all four [^{32}P]α-deoxynucleotide triphosphates and gel-purified before use. The bands migrating below SIF and GAF in Fig. 2B, lanes 5 through 8, result from binding of cellular proteins to a CAAT motif in the human SIE. They are also not ligand-inducible, nor are they competed by the GAS or high-affinity SIE oligonucleotides (*20*).
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25. The production and characterization of rabbit polyclonal antiserum anti-91 (recognizes Stat91 and Stat84) and anti-91T (recognizes only Stat91) were previously described (*14–16*). For addition to mobility-shift assays, antisera diluted in PBS were added to binding reactions and incubated for 20 to 30 min at 0°C before addition of labeled probe. Controls received PBS alone or preimmune serum diluted in PBS. The reactions were then incubated for 20 min at 25°C and subjected to mobility-shift assay using the high-affinity SIE probe as described (*10*).
26. Cytosol from spinner cultures of HeLa cells was prepared as described [J. D. Dignam, R. M. Lebovitz, R. G. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983)]. Three portions (25 μl) were each mixed with a combination of buffer D and high-salt buffer (*22*) to raise the salt and glycerol concentration (32.5 μl). To these, 1 μl of either PBS, normal rabbit serum, or anti91T was added, and the reactions were incubated overnight at 4°C. Immune complexes were collected by incubation with protein G–agarose, and depleted or mock-depleted supernatants were used in an in vitro activation assay. Briefly, each supernatant was divided into two equal portions, mixed with detergent-solubilized A431 membranes in the absence or presence of EGF, and then incubated for 30 min at 30°C in the presence of kinase buffer (*10*). One-half of each activation reaction was subjected to mobility-shift assay as described (*10*). The 2fTGH, U3, and C91 cells lines were cultured in DMEM with 10% calf serum and G418 (200 μg/ml of active drug) and grown to confluence in this medium before extract preparation. Cytoplasmic extract from each of these cell lines was prepared as described (*19*) and subjected to an in vitro activation assay in the presence or absence of EGF as described (*10*), except that activation was performed at 15°C instead of 30°C, which facilitates the detection of SIF-A in these extracts (*20*).
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28. Cultures of A431 cells were incubated at 0°C for 20 min and activated with EGF (100 ng/ml) for various lengths of time at 0°C. Activation at 0°C was done to slow the kinetics of activation and nuclear translocation. Cytoplasmic and nuclear extracts were prepared as described (*19, 22*), except that crude nuclei were resuspended in hypotonic buffer and pelleted through 30% sucrose cushions before extraction with high-salt buffer. Cytosolic and nuclear extracts (12 μg) from each treatment were subjected to mobility-shift analysis with labeled high-affinity SIE probe (*10*). For protein immunoblot analysis, equal amounts of each extract (30 μg) were denatured in Laemmli buffer, and proteins were separated by SDS-PAGE (7.5%) and transferred to nitrocellulose in a semi-dry transfer apparatus. The membrane was blocked, incubated with anti-91T serum (1:2000), and bound antigen-antibody complexes were detected with horseradish peroxidase–conjugated donkey antibody to rabbit immunoglobulin G (1:3000) and enhanced chemiluminescence (Amersham).
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30. Addition of antibody to phosphotyrosine after SIF activation in vitro did not affect SIF C complex formation (*10*). Our ability to detect phosphotyrosine in SIF in the present experiment can be attributed to several factors. First, in vitro–activated A431 extracts contain an extremely high concentration of tyrosine-phosphorylated proteins, which compete for binding to antibody. To eliminate this problem, we used nuclear extracts from HER14 cells activated in vivo; these fractions contain much less phosphotyrosine. In addition, incubation with antibody was for 2 hours at 0°C and 0.5 hours at 25°C before the addition of labeled SIE probe. We also used higher antibody concentrations.
31. Nuclear extracts from EGF-treated A431 cells

were prepared (*19, 22*) in the absence of phosphatase inhibitors; 30 mM KCl was included to maintain equivalent osmolarity, and 1.5 mM $MgCl_2$ was added to stabilize nuclei. The protein tyrosine phosphatase (PTPase) used in this experiment was a bacterially expressed T cell PTPase [D. E. Cool *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5257 (1989)], purified to homogeneity. Briefly, portions of the nuclear extracts (3 μl, 12 μg of protein) received either 1 μl of 10 mM vanadate or 1 μl of water and then either 2 μl of PTPase buffer [25 mM imidazole (pH 7.0), 0.2% 2-mercaptoethanol, and BSA (1 mg/ml) or 2 μl of fivefold serial dilutions of the PTPase in this buffer. Reactions were incubated for 30 min at 37°C and then placed on ice. Water (1 μl) was added to reactions that had received sodium vanadate before incubation, and 1 μl of sodium vanadate was added to the others (so that all reactions contained 0.5 mM sodium vanadate before mobility-shift assay). These reactions were then subjected to mobility-shift assay with high-affinity SIE probe (*10*).

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33. Antibodies to Stat113 (*14, 15, 17*) do not react with any SIF complexes (*20*).
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36. We thank members of our labs for advice and discussion; T. Grodzicker and B. Stillman for comments on the manuscript; G. Stark and I. Kerr for the U3A cells and communicating unpublished data; and D. Barford (Cold Spring Harbor Laboratory) for PTPase. H.B.S. was supported by an NIH postdoctoral training grant (CA09311) and by an Andrew Seligson Memorial Postdoctoral Fellowship. K.S. was supported by a Cancer Research Institute postdoctoral fellowship. Supported by NIH grant CA45642 (to M.Z.G.) and AI32489 (to J.E.D.).

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A Single Phosphotyrosine Residue of Stat91 Required for Gene Activation by Interferon-γ

Ke Shuai, George R. Stark, Ian M. Kerr, James E. Darnell Jr.*

Interferon-γ (IFN-γ) stimulates transcription of specific genes by inducing tyrosine phosphorylation of a 91-kilodalton cytoplasmic protein (termed STAT for signal transducer and activator of transcription). Stat91 was phosphorylated on a single site (Tyr^{701}), and phosphorylation of this site was required for nuclear translocation, DNA binding, and gene activation. Stat84, a differentially spliced product of the same gene that lacks the 38 carboxyl-terminal amino acids of Stat91, did not activate transcription, although it was phosphorylated and translocated to the nucleus and bound DNA. Thus, Stat91 mediates activation of transcription in response to IFN-γ.

Signaling from a ligand-bound cell surface receptor to the nucleus can be accomplished by tyrosine phosphorylation of latent cytoplasmic transcriptional factors that are transported to the nucleus (*1, 2*). In cells treated with IFN-α, a 113-kD protein and two closely related proteins of 91 and 84 kD become phosphorylated on tyrosine, whereas after IFN-γ treatment the 91- and 84-kD proteins but not the 113-kD protein become phosphorylated on tyrosine. After activation the proteins are translocated to the nucleus where they take part in transcriptional activation (*1, 2*). We refer to this group of tyrosine kinase substrates as STAT proteins.

The Stat91 phosphoprotein can, by itself, recognize the IFN-γ–activated site (GAS), a DNA element required for immediate gene induction by IFN-γ (*2–5*). We identified the single tyrosine in Stat91 that is phosphorylated in response to IFN-γ and found that this residue is required for the Stat91-dependent gene activation by cotransfection assays. The protein Stat84, a product of differential splicing that lacks the COOH-terminal 38 amino acids of Stat91 (*6*), became phosphorylated on the same site, was translocated to the nucleus, and bound to GAS, but did not activate transcription. We conclude that phosphorylated Stat91 acts as the primary positive-acting transcription factor in response to IFN-γ.

K. Shuai and J. E. Darnell Jr., Rockefeller University, Laboratory of Molecular Cell Biology, New York, NY 10021.
G. R. Stark, Cleveland Clinic Foundation Research Institute, Cleveland, OH 44195.
I. M. Kerr, Imperial Cancer Research Foundation, Lincoln's Inn Fields, London, United Kingdom.
*To whom correspondence should be addressed.

Thermolysin digestion of ^{32}P-labeled Stat91 from IFN-γ–treated cells yielded a single peptide labeled on tyrosine (*2*). The Stat91 protein contains 19 tyrosines (6), and to determine the location of the phosphorylated residue or residues, we examined a tryptic digest of ^{32}P-labeled Stat91 from IFN-γ–treated cells (Fig. 1A). IFN-γ induced phosphorylation of a single tryptic peptide (X) on tyrosine. Peptide X was recovered and stepwise Edman degradation done. The labeled phosphotyrosine was released in the fourth degradative cycle (Fig. 1B). Computer alignment of all the potential tryptic peptides showed a single peptide [amino acids 698 to 703 (*7*)] in which tyrosine was the fourth amino acid, revealing this peptide as the major candidate for IFN-γ–stimulated tyrosine kinase action (Fig. 1C). The putative phosphorylated peptide contained a single tyrosine at residue 701.

We prepared a synthetic peptide corresponding to amino acids 693 to 707. This peptide was exposed to purified $p43^{v\text{-}abl}$

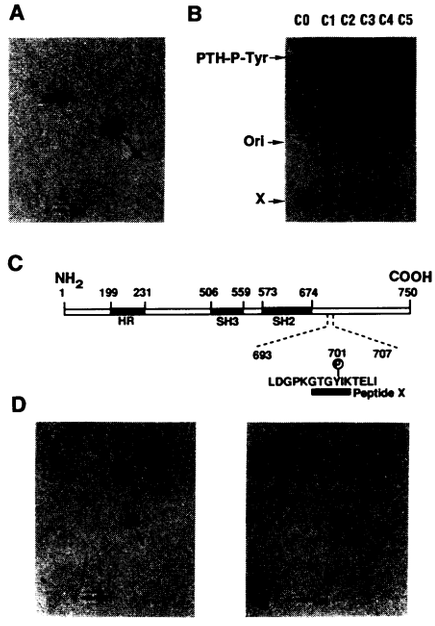

Fig. 1. Identification of the phosphotyrosine residue in Stat91. (**A**) Tryptic phosphopeptide map of ^{32}P-Stat91 from IFN-γ–treated FS2 cells (*16*). Phosphoamino acid analysis indicated that only peptide X contains phosphotyrosine (*2*). (**B**) Edman degradation of peptide X (*14*). The position of the PTH-P-Tyr marker detected by ultraviolet light is indicated. (**C**) Schematic diagram showing the site of the phosphotyrosine residue in Stat91. HR, heptapeptide repeat; SH2, *Src* homology domain 2; and SH3, *Src* homology domain 3. (**D**) The synthetic peptide LDGPKGTGYIKTELI which was phosphorylated with ^{32}P-labeled tyrosine was digested with trypsin and analyzed by two-dimensional peptide mapping either alone (left panel) or mixed with the same amount of ^{32}P-labeled peptide X (right panel). Ori, origin. The synthetic peptide (10 μg) (obtained from Genetics) was incubated with 1 U of $p43^{v\text{-}abl}$ (Oncogene Science), in 50 mM Hepes (pH 7.4), 0.1 mM EDTA, 0.015% Brij 35, 0.1 mM ATP, 10 mM $MgCl_2$, and 2 μCi of [γ-^{32}P]ATP for 30 min at 30°C. The ^{32}P-labeled peptide was subjected to electrophoresis at pH 3.5 on a thin-layer chromatography plate and purified. Tryptic digestion of ^{32}P-labeled peptide was done as described (*14, 15*). L: Leu; D: Asp; G: Gly; P: Pro; K: Lys; T: Thr; Y: Tyr; I: Ile; E: Glu.