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

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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-

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tivity was undetectable after 1 hour of PDGF treatment, whereas it was still detectable for up to 6 hours in response to IFN-γ. In addition, SIF activation occurred in cells treated with PDGF at 4°C, but IFN activation required a 37°C incubation (*20*).

We tested the specificity of SIF activation in cell lines overexpressing growth factor receptors (Fig. 1D). Colony stimulating factor–1 (CSF-1) stimulated the DNA binding activity of SIF in fibroblasts expressing the CSF-1 receptor (*21*). Acidic and basic FGFs and k-FGF failed to activate SIF in FGF-responsive Swiss 3T3 cells (*20*) or in WT7 cells (*22*) that overexpressed the type 1 FGF receptor (*flg*) gene, and insulin treatment did not activate SIF in cells overexpressing the human insulin receptor (*23*). Despite the absence of SIF stimulation by FGFs and insulin, all these growth factors caused receptor autophosphorylation and an increase in tyrosine phosphorylation of cellular proteins (*20*).

Comparison of the SIF DNA sequence with those of IFN-γ response elements (*24*) suggested that the former might serve as a binding site for p91. To test this possibility, we treated band shift reactions with antibodies to the different components of ISGF-3 (Fig. 1E). The PDGF-stimulated complex was unaffected by antibodies to the p48 and p113 components of ISGF-3, but DNA binding activity was abrogated by antibodies to p91 (anti-p91), concomitant with the appearance of a more slowly migrating supershifted complex. Specificity of DNA binding was demonstrated by competition with SIF-binding sequences but absence of competition by an irrelevant sequence (the interferon-stimulated response element). Similar involvement of p91 could be demonstrated for the binding activity stimulated by both IFN-α and IFN-γ.

To test the involvement of tyrosine phosphorylation in p91 activation, we labeled cells with [^{32}P]orthophosphate and treated them with IFNs or growth factors (*25*). Immunoprecipitation of labeled extracts with anti-p91 showed a stimulation of phosphorylation of this protein in response to IFN-α, IFN-γ, and PDGF (Fig. 2A). A protein the size of the 113-kD subunit of ISGF-3 coprecipitated with p91 in extracts from IFN-α–treated cells. However, no associated protein was detected in samples from IFN-γ–treated or PDGF-treated cells. This reflects the exclusive activation of p113 in response to IFN-α, whereas p91 phosphorylation occurs in response to all three ligands. Phosphoamino acid analysis revealed the presence of phosphotyrosine in p91 from extracts of treated cells, whereas samples from untreated cells contained only phosphoserine (Fig. 2B). Peptide map analysis of tryptic digestion products revealed phosphorylation of the same single new tryptic phosphopeptide in response to all three agents (Fig. 2C).

Tyrosine phosphorylation of p91 in response to growth factors was also detected by immunoblotting with antibodies to phosphotyrosine (anti-pTyr) (Fig. 2D). A 91-kD protein immunoprecipitated by anti-p91 from IFN- or EGF-treated cells, but not from cells treated with FGF, was detected by anti-pTyr (*26*). Thus, the 91-kD subunit of ISGF-3, or an immunologically related protein, is tyrosine phosphorylated and activated in response to specific growth factors. The absence of SIF binding activity in FGF-treated cells is apparently due to the inability of FGF to stimulate tyrosine phosphorylation of p91.

The EGF receptor (EGFR) and other receptor tyrosine kinases are coupled to Ras through intermediary proteins that recognize the receptor-autophosphorylated cytoplasmic tail (*6*, *8*). Cells expressing different mutants of the human EGFR (*27*) were activated with EGF or IFN-α and tested for p91 activation (Fig. 3A). Only cells expressing the wild-type receptor showed full stimulation of p91 in response to EGF. Truncation of the receptor cytoplasmic tail to remove the major autophosphorylated tyrosines greatly diminished the ability of EGF to stimulate p91 DNA binding activity. Mutation of the five autophosphorylation sites to Phe also prevented the normal activation of p91 in response to EGF, even though a similar amount of receptor was expressed in these cells (Fig. 3B). All cells responded normally to IFN-α. These results suggest that tyrosine autophosphorylation of the receptor is required for EGF activation of p91. The p91 subunit, an SH2 domain–containing protein (*28*), could bind to phosphotyrosine residues of the EGFR. Indeed, an SH2 domain–containing protein and antibodies to phosphotyrosine prevent SIF activation in vitro (*19*).

Mutations eliminating tyrosine autophosphorylation sites on EGFR have little effect on the mitogenic properties of EGF (*27*, *29*); hence, activation of p91 may not influence mitogenesis. To further assess the connection between p91 activation and mitogenesis, we investigated the involvement of Ras in p91 action in cells expressing dominant negative mutations of H-Ras.

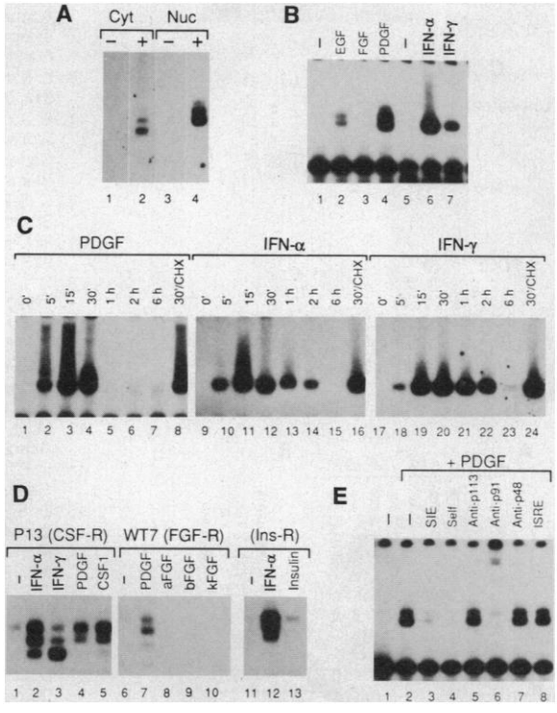

Fig. 1. Stimulation of DNA binding activity in response to growth factors. Cells were untreated or treated with the indicated growth factors and cytokines before detergent lysis and analysis of extracts by gel band shift with a labeled oligonucleotide containing an SIF recognition sequence (*18*). (**A**) Swiss 3T3 mouse fibroblasts were treated with human recombinant PDGF-BB (40 ng/ml) for 15 min and fractionated into cytoplasmic (Cyt) and nuclear (Nuc) extracts. Cytoplasmic (5 μg) or nuclear (10 μg) extracts were added to DNA binding reactions. (**B**) Human FS2 fibroblasts were treated with human recombinant EGF (250 ng/ml), FGF (50 ng/ml), PDGF (40 ng/ml), IFN-α (500 U/ml), or IFN-γ (1 ng/ml) for 15 min, as indicated. (**C**) Human FS2 fibroblasts were treated for the indicated times with PDGF, IFN-α, and IFN-γ. Cells treated with cycloheximide (CHX; 50 μg/ml) (lanes 8, 16, and 24) were first treated with CHX for 15 min, and then CHX was present throughout stimulation. (**D**) P13 cells overexpressing the human CSF-1 receptor (CSF-R) (lanes 1 through 5), WT7 cells overexpressing the *flg* FGF receptor (FGF-R) (lanes 6 through 10), and 3006 cells expressing the human insulin receptor (Ins-R) (lanes 11 through 13) were treated for 15 min, as indicated, with the following ligands: murine IFN-α (5000 U/ml); murine IFN-γ (5 ng/ml), human PDGF-BB (50 ng/ml), human CSF-1 (10,000 U/ml), acidic (aFGF), basic (bFGF), and k-FGF (200 ng/ml), or bovine insulin (1 μg/ml). Weak DNA binding activity in lane 13 was not reproducibly stimulated in response to insulin. (**E**) Extracts from human FS2 fibroblasts treated for 15 min with PDGF were analyzed by band shift with a modified SIF binding sequence recognizing SIF with higher affinity (*20*). Competitions were carried out with 100-fold molar excess of unlabeled oligonucleotide, as indicated. Antibodies to human p113, p91 (*11*), or p48 (*35*) were added at a final concentration of 1:200 for lanes 5 through 7, respectively. SIE, sis-inducible element.

A Ser-to-Asn mutation at position 17 of H-Ras (N^{17}-Ras) inhibits Ras activation in a dominant fashion that suppresses mitogenic responses (*30*). In both parental and N^{17}-Ras–expressing cells (*31*), p91 DNA binding activity was stimulated by PDGF (Fig. 3C), in spite of the abundant amounts of mutant Ras protein expressed. However, N^{17}-Ras prevented the Ras-mediated activation of proteins the size of MAP kinase (Fig. 3D), an essential step in PDGF-induced mitogenesis.

Our findings point to a distinct mechanism for growth factor signaling, directly linking receptor tyrosine kinase activation to stimulation of transcription factors. It is not clear what enzyme phosphorylates p91 in response to growth factors. Activation of p91 in response to PDGF at 4°C suggests close proximity to the PDGF receptor. The tyrosine kinase Tyk2 is necessary for IFN-α signaling (*32*), and the related enzymes Jak1 and Jak2 appear to mediate IFN-γ action (*20*). Cytoplasmic tyrosine kinases may also be necessary for growth factor stimulation of p91.

It is known that receptor tyrosine kinases can stimulate cells to proliferate or to differentiate and withdraw from the cell cycle depending on the cellular context and specific culture conditions (*33*). It has been difficult to reconcile the variety of outcomes of these signaling pathways with a uniform function for Ras. Perhaps differential activation of distinct sets of genes after the activation of ligand-specific sets of latent transcription factors modulates the outcome of a simultaneous, Ras-mediated common signal.

A lack of specificity would appear to result from multiple signaling pathways impinging on a single transcription factor, ISGF-3 p91. Both IFN-α and IFN-γ activate distinct sets of genes in spite of stimulating tyrosine phosphorylation of p91 (*12, 24, 34*). However, only IFN-α leads to the additional tyrosine phosphorylation of the 113-kD transcription factor subunit (*11*), whereas IFN-γ and PDGF activate only p91. Assembly of activated p91 and p113 into the ISGF-3α complex after IFN-α treatment allows the association of this complex with the 48-kD ISGF-3γ subunit (*14, 15*), a protein with a distinct DNA binding specificity that directs the complex to a regulatory element unique to IFN-α–stimulated genes (*35, 36*). A similar specificity-determining subunit may be involved in each signaling pathway from a distinct receptor, combining with common p91 subunits and directing the complex to appropriate target genes.

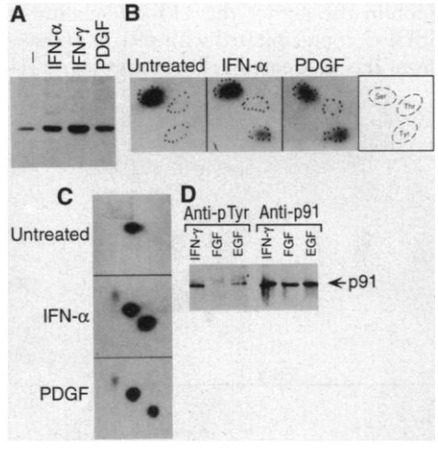

Fig. 2. Tyrosine phosphorylation of p91 by PDGF and IFNs. (**A**) Immunoprecipitation of proteins from extracts of [^{32}P]orthophosphate-labeled cells. Human FS2 fibroblasts were labeled for 2 hours before stimulation with the indicated factors for 10 min. Proteins immunoprecipitated with anti-p91 were analyzed by SDS-PAGE (*25*). (**B**) Phosphoamino acid analysis. Labeled p91 was excised from gels, hydrolyzed, and analyzed by thin-layer chromatography. Migration of phosphoamino acid standards is indicated at right. (**C**) Tryptic phosphopeptide mapping of p91. Immunoprecipitated p91 from [^{32}P]orthophosphate-labeled cells untreated or treated with IFNs or PDGF as indicated were digested with trypsin and analyzed by two-dimensional thin-layer electrophoresis and chromatography. With longer exposure, a fourth phosphopeptide fragment was detected in both unstimulated and stimulated samples. (**D**) FGF did not induce tyrosine phosphorylation of p91. Immunoprecipitated p91 was analyzed by SDS-PAGE and immunoblotting with anti-pTyr or anti-p91 (*26*).

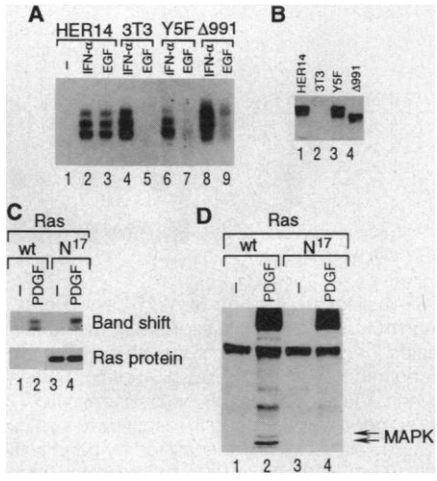

Fig. 3. Requirement of receptor autophosphorylation but not Ras activation for activation of p91. (**A**) Transfected mouse cells stably expressing a wild-type human EGF receptor (HER14, lanes 1 through 3), no human receptor (3T3, lanes 4 and 5), mutant receptors having the five major phosphotyrosine sites converted to Phe (Y5F, lanes 6 and 7), or a mutant receptor truncated in the cytoplasmic tail at amino acid 991 (Δ991, lanes 8 and 9) were treated with mouse IFN-α (2500 U/ml) or human EGF (250 ng/ml) for 15 min, as indicated, and nuclear extracts were analyzed by band shift. (**B**) Detergent lysates of the same cell lines were analyzed by immunoblotting for the presence of the human EGF receptor with rabbit antibody to EGFR (anti-EGFR) (*38*). (**C**) Rat L6 myoblasts (lanes 1 and 2) or L6 cells stably transfected with dominant negative N^{17}-Ras under a hormone-responsive promoter (*31*) and induced with 1.5 μM dexamethasone overnight (lanes 3 and 4) were treated with PDGF, as indicated. Extracts were analyzed for p91 activation by band shift (top) or for Ras expression by immunoblotting (bottom). (**D**) Extracts from (C) were analyzed for phosphorylation of MAP kinase (MAPK) by immunoblotting with anti-pTyr.

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18. We prepared and analyzed protein extracts from cells treated with various cytokines in gel band shift assays as described (*14, 15*) using as probe a ^{32}P-labeled, double-stranded oligonucleotide with the sequence 5′-GATCAGCTTCATTTCCCG-TAAATCCCTA-3′ (*16*) or a modified oligonucleotide sequence binding the same proteins with higher apparent affinity (*20*). Cells were incubated in medium containing 0.5% serum for 24 to 48 hours before treatment with cytokines.
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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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A Common Nuclear Signal Transduction Pathway Activated by Growth Factor and Cytokine Receptors

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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

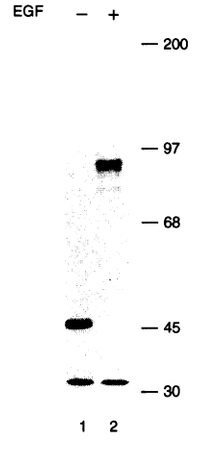

Fig. 1. DNA-affinity precipitation analysis of SIE-binding proteins from untreated and EGF-treated A431 cells. A431 cells were labeled for 3 hours with [^{35}S]methionine (200 μCi/ml) and treated with either PBS (lane 1) or recombinant human EGF (100 ng/ml, lane 2) for 15 min. Cytoplasmic extracts were 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).

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