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A Growth Factor-Induced Kinase Phosphorylates the Serum Response Factor at a Site That Regulates Its DNA-Binding Activity

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A signaling pathway by which growth factors may induce transcription of the c-*fos* proto-oncogene has been characterized. Growth factor stimulation of quiescent fibroblasts activates a protein kinase cascade that leads to the rapid and transient phosphorylation of the serum response factor (SRF), a regulator of c-*fos* transcription. The in vivo kinetics of SRF phosphorylation and dephosphorylation parallel the activation and subsequent repression of c-*fos* transcription, suggesting that this phosphorylation event plays a critical role in the control of c-*fos* expression. The ribosomal S6 kinase pp90^{rsk}, a growth factor-inducible kinase, phosphorylates SRF in vitro at serine 103, the site that becomes newly phosphorylated upon growth factor stimulation in vivo. Phosphorylation of serine 103 significantly enhances the affinity and rate with which SRF associates with its binding site, the serum response element, within the c-*fos* promoter. These results suggest a model in which the growth factor-induced phosphorylation of SRF at serine 103 contributes to the activation of c-*fos* transcription by facilitating the formation of an active transcription complex at the serum response element.

Growth factor stimulation of fibroblasts triggers a highly regulated series of events that induces the reentry of resting cells into the cell cycle, leading to DNA replication and cell division. A critical step in the mitogenic response is the rapid and transient transcriptional activation of a group of 50 to 100 genes termed immediate-early genes (IEGs) (reviewed in reference 37). One of the best-characterized IEGs is the c-*fos* proto-oncogene. Transcription of c-*fos* and other IEGs is induced within minutes of growth factor stimulation and is then rapidly repressed to prestimulation levels by 60 min (2, 16, 33, 44). This rapid pulse of IEG expression suggests that the products of these genes may have a regulatory role in the cellular response to growth factors. This idea is supported by the finding that a number of IEGs, including c-*fos*, encode transcription factors whose expression is correlated with progression through the cell cycle (reviewed in references 17 and 37).

A key question that remains to be resolved is how a growth factor-induced signal is transduced from the plasma membrane to the nucleus to regulate the expression of c-*fos* and other IEGs. The observation that IEG transcriptional activation occurs without the need for new protein synthesis (31, 44) has raised the possibility that the induction signal is propagated via the activation of a kinase cascade. In contrast, the subsequent repression of IEG transcription has been shown to be dependent on new protein synthesis (31, 44), suggesting that a transcriptional repressor must be newly synthesized upon growth factor stimulation.

A 20-bp region of dyad symmetry, known as the serum response element (SRE), is the major determinant of the transcriptional response of c-*fos* to serum and purified growth factors (22, 28, 32, 73, 74). The SRE, which is centered 310 bp upstream of the start site of c-*fos* transcription, specifically binds a 67-kDa protein known as the serum response factor (SRF) (28, 32, 59, 74). The ability of SRF to bind to mutant SREs in vitro correlates directly with the ability of the SRE to confer serum responsiveness in vivo (reviewed in reference 62). Furthermore, mutational analysis of the SRE has shown that a minimal SRF-binding site is sufficient to mediate rapid and transient kinetics of transcription in response to serum stimulation (63). These results, taken with the finding that a number of IEGs in addition to c-*fos* contain binding sites for SRF within their regulatory regions (12, 43, 60), suggest that SRF plays an important role in both the activation and repression of IEG transcription.

In addition to SRF, several other factors which interact with the c-*fos* SRE have been identified (3, 7, 34, 53, 64). One of these proteins, p62/ternary complex factor, is likely to be encoded by the *elk*-1 gene (39, 61), a member of a family of genes related to the c-*ets* proto-oncogene (41). Elk-1 cannot bind to the SRE on its own, but interacts with SRF when it is bound to the SRE to form a ternary complex (67, 68). In certain cell types, binding of Elk-1 appears to be important for the activation of c-*fos* in response to protein kinase C-dependent signals (30). However, the exact roles that Elk-1 and other c-*fos* SRE-binding factors play in the regulation of IEG transcription remain to be determined.

Since a variety of studies indicate that SRF is a critical component of the pathway by which growth factors activate and repress transcription, recent work has focused on identifying posttranslational modifications of SRF that may regulate its ability to control IEG transcription. SRF has been shown to be phosphorylated in vitro by casein kinase II (CKII) at a site that is also phosphorylated in vivo (40, 47, 48, 51). However, it is not yet known whether enhanced phosphorylation of SRF at the casein kinase II site occurs upon growth factor stimulation with kinetics that are consistent with this modification playing a role in the rapid activation of c-*fos* transcription.

To determine whether a growth factor-induced modifica-

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tion of SRF might play a role in the activation of IEG transcription, we analyzed the in vivo phosphorylation state of SRF. We find that SRF becomes transiently phosphorylated at Ser-103 within minutes of growth factor stimulation with kinetics that parallel the induction and repression of IEG transcription. Furthermore, phosphorylation at this site enhances the rate and affinity with which SRF associates with the SRE in vitro. We have identified a growth factor-inducible kinase, $pp90^{rsk}$, that phosphorylates SRF in vitro at the same serine residue that is inducibly modified in vivo upon growth factor stimulation. Taken together, these results suggest that one mechanism by which growth factors stimulate IEG transcription is through the activation of a kinase cascade that transduces growth factor signals to the nucleus, resulting in the phosphorylation of SRF and an alteration in its binding activity.

MATERIALS AND METHODS

Cell culture and biosynthetic labeling. Swiss 3T3 and COS cells were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 10% fetal calf serum (GIBCO). Swiss 3T3 cells were made quiescent by growing them to 90% confluence and then replacing the growth medium with DMEM containing 0.5% calf serum (GIBCO) and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4) for 12 to 24 h. To label with $^{32}P_i$ (carrier free; ICN), serum-starved Swiss 3T3 cells were further cultured for 12 to 16 h in phosphate-free DMEM (ICN) containing 0.5% dialyzed calf serum and 2.5 mCi of $^{32}P_i$ per ml (4 ml per 100-mm-diameter dish). Cells were then stimulated for the desired amount of time by the addition of 20% dialyzed fetal calf serum, 25 ng of epidermal growth factor (EGF) per ml, or 100 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml.

COS cells were transfected with 3 μg of SRF expression vector per 100-mm-diameter dish by the DEAE-dextran procedure (4). To label with $^{32}P_i$, 36 h after transfection, cells were cultured for 4 h in phosphate-free DMEM containing 0.3 mCi of $^{32}P_i$ per ml (2.5 ml per 60-mm-diameter dish).

To prepare 3T3 and COS cell lysates, the labeling medium was removed, and the cells were rinsed briefly with STE buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA) and lysed by the addition of a boiling solution of sodium dodecyl sulfate (SDS) buffer (0.5% SDS, 50 mM Tris-HCl [pH 7.4]). The lysate was boiled for 5 min and then diluted by the addition of 4 volumes of 1.25× radioimmunoprecipitation assay (RIPA) buffer without SDS (1× RIPA is 150 mM NaCl–50 mM Tris-HCl [pH 7.4]–1% Nonidet P-40 [NP-40]–0.5% deoxycholate containing 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 50 mM β-glycerophosphate [pH 7.2], 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EDTA, and 2 mM dithiothreitol [DTT]).

Lysates were vortexed vigorously for 30 s and then cleared by centrifugation at 10,000 × *g* for 30 min. The cleared lysates were used for immunoprecipitations with anti-N-SRF antibodies that were raised against a bacterially expressed TrpE-SRF-N fusion protein which contains the amino-terminal 45 to 245 amino acids of SRF as described previously (54). Nonspecific and specific competitions were performed by preincubating the anti-N-SRF antibodies with TrpE and TrpE-SRF-N proteins, respectively.

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis. Following fractionation by SDS-polyacrylamide gel electrophoresis (PAGE), ^{32}P-labeled proteins were transferred to nitrocellulose and visualized by autoradiography. The portion of the filter containing SRF was excised, soaked in 0.5% polyvinylpyrrolidone-360 in 100 mM acetic acid for 30 min at 37°C, washed with H_2O, and then washed with freshly made 50 mM ammonium bicarbonate. The protein was digested at 37°C overnight by placing the membrane in 200 μl of 50 mM ammonium bicarbonate containing 10 μg of trypsin (Worthington), followed by the addition of another 10 μg of trypsin and a 2-h incubation at 37°C. Digested protein was lyophilized and oxidized as described previously (46). Tryptic peptides were separated on thin-layer cellulose plates by electrophoresis at pH 1.9 (acetic acid–88% formic acid–H_2O [156:50:1,794 {vol/vol/vol}]) for 40 min at 1.0 kV in the first dimension, followed by ascending chromatography in a buffer composed of *n*-butanol, pyridine, acetic acid, and H_2O (75:50:15:60 [by volume]) for 7 h in the second dimension (8). Labeled tryptic phosphopeptides were detected by autoradiography.

To perform phosphoamino acid analysis, a portion of the lyophilized peptides was resuspended in boiling HCl (5.7 M) and incubated at 110°C for 60 min. The samples were then lyophilized and resolved by electrophoresis, using pH 1.9 buffer in the first dimension and pH 3.5 buffer (acetic acid–pyridine–H_2O [10:1:189 {vol/vol/vol}]) in the second dimension, as described previously (8).

Preparation of recombinant SRF in bacteria and COS cells. To mutate Ser-103 to alanine, a 450-bp *Xma*III-to-*Pst*I fragment from the human SRF clone, pT7ΔATG (56), was inserted into M13mp18. Site-directed mutagenesis (66) was performed with the following oligonucleotide: TCCATCT CGGCCAGGCTCCG. The Ser-103 mutation was incorporated into TrpE-SRF-N, which contains amino acids 46 to 245 of human SRF fused to TrpE (54), and T7ΔATG by replacement of a 300-bp *Sac*II-to-*Stu*I fragment with the corresponding fragment from the mutated M13mp18. pARSRF.Nde (47) was used to overexpress the full-length SRF in bacteria (bSRF). To overexpress SRF mutated at Ser-103 (SRFm103), the 287-bp *Sma*I fragment of pARSR F.Nde was replaced with the corresponding fragment from the mutated pT7ΔATG plasmid. Wild-type SRF and SRFm103 proteins were overexpressed in bacteria and gel purified as described previously (48, 79). When the entire lysate was used as a source of bSRF in kinase reactions, the lysate was first heated at 80°C for 10 min to inactivate endogenous kinases.

To overexpress SRF and SRFm103 in COS cells, a 1.6-kb *Hin*dIII-*Xba*I fragment from the corresponding T7ΔATG plasmid was inserted into *Hin*dIII- and *Xba*I-digested pSG424 (65) so that expression of SRF was directed by the simian virus 40 early gene promoter. A vector directing the expression of SRF mutated at four serine residues between amino acids 77 and 85 [SRFm(77-85) (48)] was provided by Ron Prywes.

Preparation of SRF in baculovirus-infected cells. To construct a vector that overexpresses human SRF in baculovirus-infected cells (pVL:SRF), pT7ΔATG was digested with *Hin*dIII and blunt ended, and *Bam*HI linkers were added. The 1.6-kb fragment containing SRF was released by digestion with *Bam*HI and ligated in to *Bam*HI-cut pVL941 (45), a baculovirus expression vector. Recombinant virus was generated by standard protocols (4). Expression of SRF in infected cells was detected by Western blot (immunoblot) analysis (54).

To purify SRF overexpressed in baculovirus cells (bvSRF), 1.5×10^8 *Spodoptera frugiperda* Sf9 cells were

infected with recombinant virus at a multiplicity of infection of 10. At 42 h postinfection, cells were lysed on ice for 5 min in 3 volumes of lysis buffer (20 mM HEPES [pH 7.9]–20% glycerol–0.05% NP-40–0.3 M KCl–0.2 mM EDTA containing 2 mM DTT, 1% NP-40, 0.5 mM PMSF, 1 μg of pepstatin per ml, 1 μg of leupeptin per ml, 100 U of trasylol per ml, 0.1 mM sodium orthovanadate, 0.1 mM ammonium molybdate, and 50 mM sodium fluoride) and then centrifuged at 100,000 × *g*. Clarified extracts were diluted 1:10 with lysis buffer lacking NP-40 (0.1%, final concentration). Extracts were passed over an SRE affinity column (75) that was prewashed with buffer C (20 mM HEPES [pH 7.9], 20% glycerol, 0.3 M KCl, 0.2 mM EDTA, 2 mM DTT, 0.5 mM PMSF). The column was washed stepwise with 0.5, 0.75, 1.5, and 2.0 M KCl in buffer C. bvSRF eluted predominantly in the 1.5 M KCl fraction, as assayed by mobility shift analysis. The 1.5 M eluate was concentrated and dialyzed in a Centricon-30 in 20 mM HEPES (pH 7.9)–10% glycerol to a final KCl concentration of 50 mM. bvSRF that was obtained by this method was approximately 95% pure, as determined by Coomassie staining. Approximately 10 mg of bvSRF was recovered per liter of cells. bvSRF was dephosphorylated by treatment with calf intestinal alkaline phosphatase and repurified as described by Marais et al. (51).

Generation of antibodies specific for Ser-103-phosphorylated SRF. To generate a Ser-103-phosphorylated SRF peptide, 2.6 mg of peptide (SRF amino acids 95 to 106; Arg-Arg-Gly-Leu-Lys-Arg-Ser-Leu-Ser-Glu-Met-Glu) was incubated with 1 μg of purified Ca^{2+}/calmodulin-dependent protein (CaM) kinase II (described below) in 100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.4)–10 mM $MgCl_2$–2 mM ATP–5 mM DTT–4 μg of calmodulin per ml–1 mM $CaCl_2$ for 4 h at 25°C. Enzyme (1 μg) was added five times and fresh ATP and calmodulin were added once over a 24-h period. The reaction mixture was then desalted by size exclusion chromatography (Sephadex G-25), and the enzyme was removed by ultrafiltration (Centricon-10). More than 90% of the peptide was phosphorylated on Ser-103, as determined by peptide sequencing (J. Lee, Molecular Biology Core Facility, Dana-Farber Cancer Institute). The phosphorylated peptide was coupled to keyhole limpet hemocyanin under conditions that favor coupling through the amino-terminal α-amino group (35a) by incubation with a limiting amount of glutaraldehyde (2 mM) in ammonium acetate buffer (pH 7.0). Rabbits were injected with the phosphorylated peptide (250 μg) in complete Freund's adjuvant and boosted every 28 days with 250 or 500 μg of peptide in incomplete Freund's adjuvant. Serum was collected 10 days after injections, and the immunoglobulin G fraction was purified by protein A-Sepharose chromatography. Affinity columns for the purification of antibodies specific for Ser-103-phosphorylated SRF were prepared by coupling either unphosphorylated or phosphorylated peptide to Affi-Gel 10 (Bio-Rad). Immunoglobulin G was applied to the affinity column containing unphosphorylated peptide, and the flowthrough of this column was applied to the phosphopeptide column. Antibodies were eluted from each column in a buffer containing 100 mM glycine (pH 3.0) followed by a buffer containing 100 mM triethylamine (pH 11.0). Antibodies were eluted into a buffer containing 100 mM Tris (pH 8.0) and 10 mg of bovine serum albumin (BSA) per ml and subsequently dialyzed against phosphate-buffered saline (PBS). Unexpectedly, antibodies that are highly specific for Ser-103-phosphorylated SRF were found in the eluate and not the flowthrough of the column containing unphosphorylated peptide. We presume that under these conditions, the antibodies have a low affinity for the unphosphorylated peptide. Similar results have been obtained during the affinity purification of two other antibodies raised against phosphorylated peptides (28a).

Immune complex kinase assay. Immune complex kinase assays to detect $pp90^{rsk}$, the mitogen-activated protein (MAP) kinase $pp44^{mapk}$, and pp70-S6K kinase activities in Swiss 3T3 cells were performed as described previously (9), using antisera specific for $pp90^{rsk}$ (9), the MAP kinases (11), and pp70-S6K (14). Subcellular fractionation of Swiss 3T3 cells was carried out as described previously (11).

To measure $pp90^{rsk}$ kinase activity in cells infected with a baculovirus vector that over expresses $pp90^{rsk}$, 2.8×10^6 Sf9 cells on 60-mm-diameter dishes were infected with recombinant baculovirus-expressed *Xenopus* RSK (a gift from Ray Erikson [78]) at a multiplicity of infection of 10. At 40 h postinfection, cells were washed with ST buffer (150 mM NaCl, 10 mM Tris [pH 7.5]), lysed in 0.5 ml of buffer containing 10 mM Tris (pH 7.2), 1 mM EDTA, 1 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.5 mM PMSF, 10 μg of leupeptin per ml, and 10 μg of pepstatin per ml, Dounce homogenized 30 times, and clarified at 100,000 × *g*. Glycerol was then added to the supernatant to a final concentration of 10%. $pp90^{rsk}$ was immunoprecipitated from this lysate, and its activity was measured by an immune complex kinase assay.

Protein kinase assays. $pp90^{rsk}$ was partially purified from growth factor-stimulated Swiss 3T3 cells by successive chromatography on DEAE-Sephacel, sulfopropyl-Sephadex, and heparin-agarose, using buffer conditions described previously for purification of pp70-S6K (6). Kinase reactions using either partially purified $pp90^{rsk}$ or $pp90^{rsk}$ that was part of an immune complex were performed as described previously (9). Briefly, the final reaction mixtures contained 20 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 50 μM ATP (20 μCi of ATP), 3 mM β-mercaptoethanol, 0.1 mg of BSA per ml, and, where indicated, 1 μg of bSRF or bvSRF substrate. The reaction was allowed to proceed for 15 min at 30°C before being terminated by the addition of 2× SDS-PAGE sample buffer followed by heating at 90°C for 5 min. Samples were then resolved by SDS-PAGE.

CaM kinase II was purified from rat brains as described by McGuinness et al. (52). The enzyme was >95% pure and exhibited an apparent V_{max} of 3 to 6 μmol min^{-1} mg^{-1} with autocamtide-2 (35) as a substrate. To phosphorylate SRF, 5 μg of bSRF, 0.05 μg of dephosphorylated bvSRF, or ~0.2 μg of SRF present in COS cell extracts was incubated with 50 to 600 ng of CaM kinase II in a reaction mixture containing 50 mM Tris (pH 7.2), 10 mM $MgCl_2$, 600 μM calmodulin, 1 mM $CaCl_2$, 0.4 to 1.0 mg of BSA per ml, and 250 μM ATP for 30 min at 30°C. To achieve stoichiometric phosphorylation, two additional aliquots of fresh CaM kinase II were added at 30-min intervals. Mock reactions were carried out under identical conditions, using CaM kinase II that had been heat inactivated at 80°C for 10 min.

To determine the level at which COS-expressed SRF was phosphorylated at the CKII site, extracts containing ~0.3 μg of SRF were dephosphorylated or mock treated as described previously (51). Extracts were then diluted into RIPA buffer, and SRF was immunoprecipitated. Dephosphorylated SRF and mock-treated SRF were incubated with 2.5 μl of purified CKII (provided by Dan Marshak) in 50 mM morpholinepropanesulfonic acid (MOPS; pH 7.0)–10 mM $MgCl_2$–100 μM ATP–10 μCi of ATP at 30°C for 1 h. Reactions were stopped

by the addition of 2× SDS-PAGE sample buffer, boiled for 5 min, and resolved by SDS-PAGE.

DNA mobility shift assay. Swiss 3T3 cells extracts from 100-mm-diameter dishes of serum-starved and serum-stimulated cells were prepared by lysis in 200 μl of lysis buffer (10 mM HEPES [pH 7.9]–0.5% NP-40–0.1% Brij 35–0.1% deoxycholate–10 mM $MgCl_2$–1 mM EDTA–5 mM EGTA containing 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM DTT, 0.5 mM PMSF, 1 mg of pepstatin per ml, 1 mg of leupeptin per ml, 2 mg of aprotinin per ml, 10 mM sodium fluoride, 10 mM *p*-nitrophenyl phosphate, 1 μM microcystin, and 0.2 μM okadaic acid). Lysates were Dounce homogenized 30 times and cleared by centrifugation at 10,000 × *g* for 30 min. Nuclear extracts of transfected COS cells were prepared as described by Dignam et al. (19) except that the supernatant was not dialyzed.

To measure the rate with which SRF binds to the SRE, ~3 ng of bvSRF, COS cell extracts containing ~1 ng of wild-type SRF or SRFm103, or Swiss 3T3 extracts (1% of a 100-mm-diameter dish) was added to a DNA mobility shift reaction mixture consisting of 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 10% glycerol, 60 mM KCl, 0.4 mg of BSA per ml, 5 μg of pUC19 per ml, 25 μg of poly(dI-dC) per ml, and 0.5 ng of ^{32}P-end-labeled probe containing the c-*fos* SRE, cut from pUC119SRE (27) as a *Hin*dIII-*Eco*RI fragment. Reaction mixtures were incubated at room temperature for the indicated amounts of time before being loaded on a 4% nondenaturing polyacrylamide gel in 0.25× Tris-borate-EDTA. Equilibrium binding reaction mixtures were incubated for 30 min. To assay for the presence of SRF phosphorylated at Ser-103, 4 μl of anti-P-SRF antibodies (diluted in PBS containing 5 mg of BSA per ml), or PBS containing 5 mg of BSA per ml as a control, were added to equilibrium binding reaction mixtures, which were incubated further for 15 min at room temperature. To measure the rate with which SRF dissociates from the SRE, equilibrium binding reaction mixtures were incubated with a 150-fold excess of unlabeled SRE for the indicated amounts of time.

RESULTS

SRF phosphorylation is growth factor inducible. To determine whether SRF is the target of a growth factor-activated protein kinase, the phosphorylation state of SRF was examined before and after serum stimulation of Swiss 3T3 cells. SRF was immunoprecipitated from ^{32}P-labeled cells that were made quiescent and then either left untreated or stimulated with serum for 5 min. In initial experiments, it was found that the overall level of SRF phosphorylation did not change appreciably upon serum stimulation (Fig. 1A). Furthermore, phosphoamino acid analysis demonstrated that SRF is phosphorylated exclusively on serine residues both before and 5 min after serum stimulation (Fig. 1B).

To examine the possibility that the level of phosphorylation of SRF at individual sites might be altered, even though the overall level of phosphorylation does not appear to change, we generated tryptic phosphopeptide maps of SRF immunoprecipitated from quiescent cells or from cells that were serum stimulated for various periods of time. As shown in Fig. 1C, SRF from quiescent and serum-stimulated cells is phosphorylated at multiple sites, and the level of phosphorylation at most of these sites is unchanged upon serum stimulation. However, a significant increase in the level of phosphorylation of one peptide, A, occurs within 5 min of serum stimulation (Fig. 1C). The serum-induced phosphorylation of SRF is reproducibly observed and also occurs within minutes of exposure of quiescent 3T3 cells to purified EGF (Fig. 2).

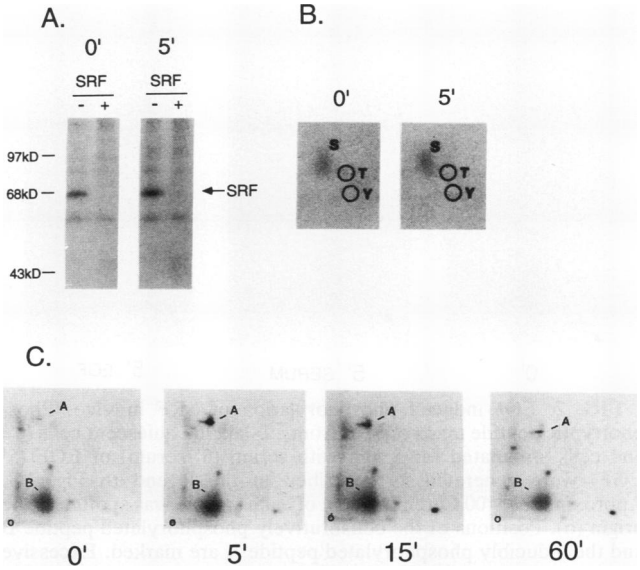

FIG. 1. Serum-induced phosphorylation of SRF in vivo. (A) SRF was immunoprecipitated from ^{32}P-labeled Swiss 3T3 cells that were quiescent (0') or serum stimulated for 5 min (5'). Immunoprecipitations were performed with anti-N-SRF antibodies in the presence (+) or absence (−) of competitor TrpE-SRF-N fusion protein. The 67-kDa SRF protein, which is immunoprecipitated only in the absence of competitor TrpE-SRF-N, is marked. (B) SRF was immunoprecipitated from ^{32}P-labeled quiescent (0') and 5-min serum-stimulated (5') cells and then subjected to phosphoamino acid analysis as described in Materials and Methods. Electrophoresis at pH 1.9 was performed from right (− [cathode]) to left (+ [anode]) and was followed by electrophoresis at pH 3.5 from bottom (−) to top (+). Positions of migration of the ninhydrin-stained phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) amino acids are indicated. (C) SRF was immunoprecipitated from ^{32}P-labeled quiescent cells that were stimulated with serum for 0, 5, 15, and 60 min, as indicated. Immunoprecipitated SRF was then digested with trypsin and processed as described in Materials and Methods. Approximately 450 Cerenkov cpm of each sample was spotted at the origin (o). The position of the tryptic peptide that is inducibly phosphorylated upon serum stimulation is indicated (A). No reproducible changes were observed in the level of phosphorylation of the other peptides, including the peptide marked B and the unmarked peptide in the bottom right corner. Electrophoresis at pH 1.9 was performed from left (+) to right (−), and chromatography was performed from bottom to top. Equivalent results were obtained in five separate experiments.

To begin to investigate the significance of the growth factor-induced phosphorylation of SRF peptide A, the kinetics of phosphorylation and dephosphorylation at this site were examined. Figure 1C shows that the increased phosphorylation of peptide A that occurs within 5 min is still detected 15 min after serum stimulation. Peptide A phosphorylation then returns to near prestimulation levels within 60 min of serum addition (Fig. 1C). Significantly, the kinetics of SRF peptide A phosphorylation and dephosphorylation parallel the kinetics of activation and repression of transcription of c-*fos* and other IEGs (2, 33). The correlation between the induced state of SRF phosphorylation and the activated state of IEG transcription, taken together with the fact that a minimal binding site for SRF can mediate both the activation and repression of c-*fos* transcription (63), suggests that the transient phosphorylation of SRF at a site contained within

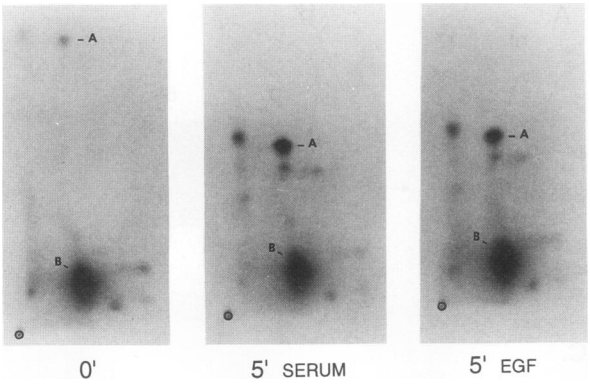

FIG. 2. EGF-induced phosphorylation of SRF in vivo. Phosphotryptic peptide maps of SRF from ^{32}P-labeled quiescent cells (0') and cells stimulated for 5 min with serum (5' serum) or EGF (5' EGF) were generated as described in the legend to Fig. 1C. Approximately 400 Cerenkov cpm of each sample was spotted at the origin (o). Positions of the constitutively phosphorylated peptide B and the inducibly phosphorylated peptide A are marked. Excessive chromatographic separation of peptides A and B, as occurred in the sample from quiescent cells, was occasionally observed.

peptide A in some way enhances the ability of SRF to activate IEG transcription.

SRF is inducibly phosphorylated on Ser-103 in vivo. To facilitate investigation of the importance of the growth factor-induced phosphorylation of SRF, we identified the amino acid that becomes newly modified. This was accomplished by generating tryptic phosphopeptide maps of wild-type SRF and SRF mutated at potential sites of phosphorylation and then determining whether a particular mutation resulted in the loss of phosphorylation on peptide A. To allow maps of exogenously introduced wild-type and mutant SRF proteins expressed in vivo to be analyzed without interference from the endogenous SRF protein, vectors directing SRF expression were transfected into COS cells. Under these conditions, immunoblotting with anti-SRF antibodies demonstrated that SRF from the transfected gene was expressed at levels 50- to 100-fold higher than the endogenous SRF level (data not shown). Initial experiments revealed that overexpression of wild-type SRF in COS cells did not alter its pattern of phosphorylation in comparison with the endogenous COS cell SRF. Furthermore, the major phosphopeptides present in serum-stimulated cells, including the inducibly phosphorylated peptide A and the constitutively phosphorylated peptide B (Fig. 1C and 2), comigrated with peptides obtained from SRF overexpressed in COS cells (Fig. 3 and data not shown).

SRF has previously been shown to be phosphorylated in vitro by CKII at several serine residues located between amino acids 77 and 85 (47). Mutation of these serines, to generate SRFm(77-85), blocks the ability of SRF to be phosphorylated in vitro by CKII (48) and in vivo when overexpressed in baculovirus-infected Sf9 cells (51) or vaccinia virus-infected HeLa cells (40). To consider the possibility that SRF might be inducibly modified at this site upon growth factor stimulation, wild-type SRF and SRFm(77-85) were overexpressed in COS cells and the tryptic phosphopeptide maps were compared. As shown in Fig. 3, mutation of the serine residues between amino acids 77 and 85 did not affect the phosphorylation of SRF on peptide A but instead blocked the phosphorylation of SRF on a site

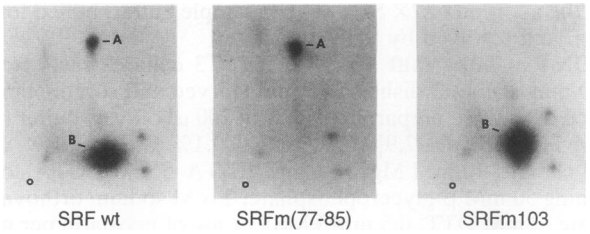

FIG. 3. SRF is inducibly phosphorylated at Ser-103 in vivo. Wild-type SRF and SRF mutants were immunoprecipitated from ^{32}P-labeled COS cells transfected with the indicated plasmids, and phosphotryptic peptide maps were generated as described in the legend to Fig. 1C. The positions of peptides A and B, which comigrate with peptides A and B observed in serum-stimulated cells, are marked.

contained within peptide B. Therefore, the growth factor-induced phosphorylation of SRF does not occur on sites previously shown to be phosphorylated in vitro by CKII. Moreover, inspection of the phosphopeptide maps shown in Fig. 1C and 2 reveals that the level of phosphorylation at the CKII site (peptide B) is not affected by serum or growth factor treatment, suggesting that phosphorylation at this site does not play a critical role in the rapid induction of IEG transcription.

To facilitate identification of the site at which SRF is inducibly phosphorylated in vivo, several additional kinases were examined for the ability to phosphorylate SRF in vitro on the tryptic peptide that is inducibly phosphorylated in vivo. This analysis identified two kinases, the ribosomal S6 kinase pp90^{rsk} (see below) and CaM kinase II (53b), that effectively phosphorylate SRF in vitro on a site contained within peptide A. pp90^{rsk} and CaM kinase II have previously been shown to phosphorylate serine residues contained within the consensus sequence, -Arg-X-X-Ser- (20, 57). Inspection of the SRF polypeptide revealed such a sequence, Arg-100–Ser–Leu–Ser-103 (56), raising the possibility that these kinases phosphorylate SRF at Ser-103 in vitro and that Ser-103 is the site of growth factor-induced phosphorylation in vivo.

To test the possibility that SRF is inducibly phosphorylated at Ser-103 in vivo, Ser-103 was changed to an alanine by site-directed mutagenesis, and the mutant SRF (SRFm103) was overexpressed in COS cells to compare its phosphotryptic peptide map with that of wild-type SRF. As shown in Fig. 3, mutation of Ser-103 blocks the ability of SRF to be phosphorylated on a site contained within peptide A, the peptide whose phosphorylation is induced upon growth factor stimulation (Fig. 1C and 2). These results suggest that growth factor stimulation of 3T3 cells results in the induced phosphorylation of SRF on Ser-103. However, additional analysis, described below, was required to rule out the possibility that SRF is inducibly phosphorylated on another residue whose phosphorylation is indirectly blocked by mutation of Ser-103 to alanine.

Generation of antibodies that specifically recognize SRF phosphorylated at Ser-103. To verify that Ser-103 is the site of growth factor-induced phosphorylation and to extend our analysis of this event, we generated antibodies that specifically recognize SRF phosphorylated at Ser-103. A peptide corresponding to amino acids 95 to 106 of SRF was synthesized and phosphorylated at Ser-103 in vitro (see Materials and Methods). This and subsequent analyses requiring the phosphorylation of an SRF substrate at Ser-103 were carried

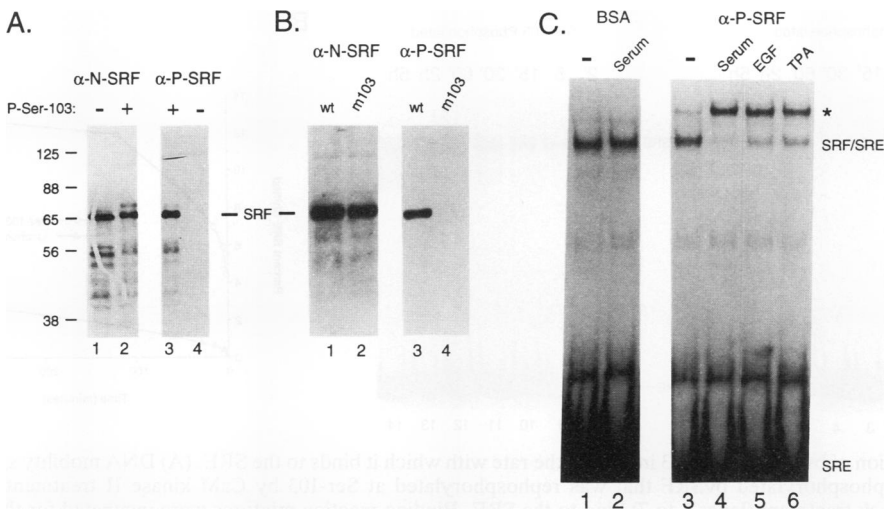

FIG. 4. Characterization of anti-P-SRF antibodies. Antibodies raised against the amino-terminal half of SRF (α-N-SRF) or the Ser-103-phosphorylated SRF peptide (α-P-SRF) were used to immunoblot bSRF that was phosphorylated at Ser-103 by incubation with CaM kinase II (+) or left unphosphorylated (−) (A) and nuclear extracts from COS cells transfected with wild-type SRF (wt) and SRFm103 (m103) (B). Sizes are indicated in kilodaltons. (C) DNA mobility shift analysis to detect the presence of SRF phosphorylated at Ser-103. Extracts from quiescent cells (−) and cells stimulated for 5 min with serum, EGF, and TPA (as indicated) were incubated with ^{32}P-labeled SRE in the presence of equivalent concentrations of BSA (lanes 1 and 2) or anti-P-SRF antibodies (lanes 3 to 6). Positions of the free probe (SRE) and the SRF-bound probe (SRF/SRE) are indicated. The asterisk marks the position of the SRF-SRE complex whose migration is further retarded by interaction with anti-P-SRF antibodies.

out by using a highly purified and active preparation of CaM kinase II that was shown to phosphorylate SRF specifically at this site (28a). Sequence analysis of the SRF peptide confirmed that CaM kinase II phosphorylated the peptide exclusively on the amino acid corresponding to Ser-103.

Antibodies (anti-P-SRF) raised against the Ser-103-phosphorylated peptide were affinity purified and tested for the ability to specifically recognize SRF phosphorylated at Ser-103. By Western analysis, the anti-P-SRF antibodies were found to recognize bSRF that was phosphorylated in vitro at Ser-103; however, these antibodies failed to recognize mock-treated, unphosphorylated SRF (Fig. 4A, lanes 3 and 4). In contrast, anti-N-SRF antibodies raised against the entire amino-terminal half of SRF (54) recognize both the unphosphorylated and Ser-103-phosphorylated forms of SRF (Fig. 4A, lanes 1 and 2). Similarly, anti-P-SRF antibodies specifically recognize wild-type SRF expressed in COS cells but not SRFm103, whereas anti-N-SRF antibodies recognize both forms (Fig. 4B). The finding that the anti-P-SRF antibody does not recognize SRFm103 expressed in COS cells, which is still phosphorylated at several serine residues (Fig. 3), coupled with our finding that SRF is the major protein recognized by the anti-P-SRF antibodies in whole-cell extracts from serum-stimulated 3T3 cells (53a), demonstrates the high degree of specificity this antibody has for SRF Ser-103.

The anti-P-SRF antibodies were next used to confirm and extend the observation that serum stimulation of 3T3 cells leads to the induced phosphorylation of SRF at Ser-103. SRF present in extracts prepared from quiescent cells and cells stimulated with serum for 5 min was detected by its ability to bind to the SRE in a DNA mobility shift assay (Fig. 4C, lanes 1 and 2). To detect the presence of SRF phosphorylated at Ser-103, anti-P-SRF antibodies were included in the DNA binding reaction. Pilot experiments using extracts from COS cells, in which wild-type SRF or SRFm103 were overexpressed, demonstrated that the anti-P-SRF antibodies bound specifically to Ser-103-phosphorylated SRF, resulting in a further retardation of the mobility of the SRF-SRE complex (see Fig. 6B). As shown in Fig. 4C, whereas only a small percentage of the SRF in quiescent cells is recognized by the anti-P-SRF antibodies (lane 3), in serum-stimulated cells, virtually all of the SRF is recognized (lane 4). This experiment demonstrates conclusively that SRF is inducibly phosphorylated at Ser-103 upon serum stimulation and suggests that essentially all of the SRF molecules in the cell become phosphorylated at this site. By this analysis, and also by Western blotting and immunostaining with the anti-P-SRF antibody (53a), the phosphorylation of SRF at Ser-103 has also been found to occur in 3T3 cells in response to EGF and TPA (lanes 5 and 6) treatment as well as in other cell types in response to various extracellular stimuli (53b, 62a).

Phosphorylation at Ser-103 enhances the binding of SRF to the SRE. We next examined the functional consequences of SRF Ser-103 phosphorylation and found that phosphorylation at this site promotes the interaction of SRF with the SRE. Purified bvSRF was first dephosphorylated by treatment with calf intestinal alkaline phosphatase. Western blot analysis confirmed that phosphatase treatment resulted in the dephosphorylation of SRF, as evidenced by an increase in its mobility on an SDS-polyacrylamide gel (data not shown). Dephosphorylated SRF was then either rephosphorylated at Ser-103 or left unphosphorylated by mock treatment. The kinetics with which these two forms of SRF associate with the SRE were examined by DNA mobility shift analysis. To measure the rate with which SRF associates with the SRE, binding reaction mixtures containing equivalent amounts of unphosphorylated or Ser-103-phosphorylated SRF were allowed to incubate with the SRE for various amounts of time before being loaded onto the gel. As shown in Fig. 5A, the phosphorylation of SRF at Ser-103 significantly increases the rate with which SRF binds to the

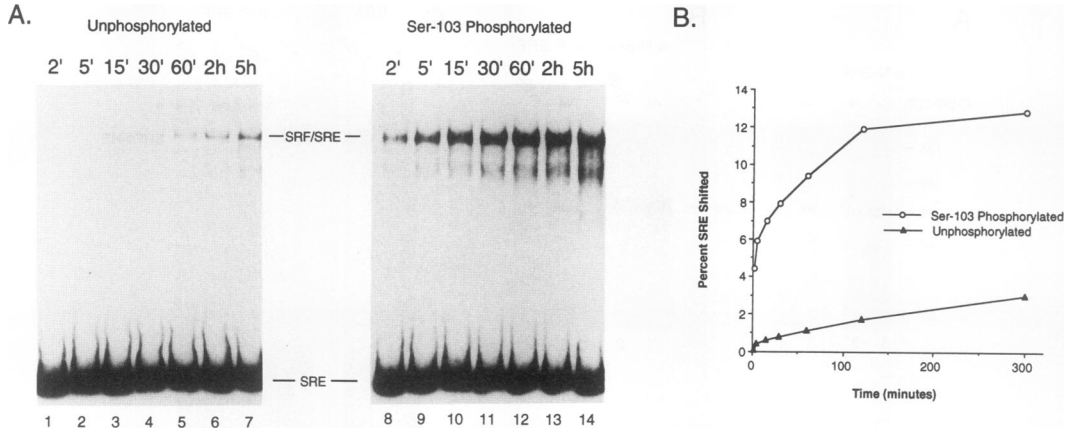

FIG. 5. Phosphorylation of bvSRF at Ser-103 increases the rate with which it binds to the SRE. (A) DNA mobility shift analysis to measure the rates with which dephosphorylated bvSRF that was rephosphorylated at Ser-103 by CaM kinase II treatment (lanes 8 to 14) or left unphosphorylated by mock treatment (lanes 1 to 7) bind to the SRE. Binding reaction mixtures were incubated for the indicated amounts of time (minutes) before being loaded on the gel. Positions of the free probe (SRE) and the SRF-bound probe (SRF/SRE) are indicated. (B) The results of the experiment shown in panel A were quantitated on a Molecular Dynamics PhosphorImager and expressed as the percentage of ^{32}P-labeled SRE probe that was bound to the SRE as a function of time.

SRE. Quantitation of these results is shown in Fig. 5B. In contrast to its effect on the association of SRF with the SRE, Ser-103 phosphorylation has little effect on the rate with which SRF dissociates from the SRE (data not shown; see Fig. 7B). Taken together, these observations suggest that Ser-103 phosphorylation also increases the equilibrium binding affinity of SRF to the SRE (Fig. 5).

Interestingly, as with Ser-103 phosphorylation, phosphorylation of SRF by CKII at serine residues between amino acids 77 to 85 also increases the rate with which SRF associates with the SRE (40, 47, 48, 51). However, in contrast to the effect of Ser-103 phosphorylation, phosphorylation by CKII also increases the rate with which SRF dissociates from the SRE and as a result does not have an overall effect on the SRF-SRE equilibrium binding affinity (51).

Our analysis in 3T3 cells has shown that SRF which becomes inducibly phosphorylated at Ser-103 upon growth factor stimulation is constitutively phosphorylated at the site phosphorylated by CKII in vitro (Fig. 1C and 2). It was therefore important to determine the effect that Ser-103 phosphorylation has on the binding of SRF to the SRE under conditions in which the CKII site is already phosphorylated. This was accomplished by comparing the binding activities of wild-type SRF and SRFm103 overexpressed in COS cells. To determine whether SRF overexpressed in COS cells is stoichiometrically phosphorylated at the CKII site, COS cell extracts containing SRF were incubated with CKII in the presence of ATP to reveal the presence of molecules previously unphosphorylated at this site. As shown in Fig. 6A, SRF overexpressed in COS cells was only weakly phosphorylated by CKII in this assay (lane 1). In contrast, SRF that was first dephosphorylated by treatment of the extracts with calf intestinal phosphatase was efficiently phosphorylated by CKII (lane 2). These results demonstrate that COS cell-expressed SRF is stoichiometrically phosphorylated at the CKII site.

To determine the extent to which SRF overexpressed in COS cells is phosphorylated at Ser-103, nuclear extracts prepared from SRF-expressing COS cells were analyzed in a DNA mobility shift assay. The addition of anti-P-SRF antibodies to the binding reaction was shown to cause a shift in the migration of the SRF-SRE complexes containing Ser-103-phosphorylated SRF. In this assay, only 25% of the wild-type SRF was found to be phosphorylated at Ser-103 (Fig. 6B). To generate SRF that was stoichiometrically phosphorylated at Ser-103, as it is in serum-stimulated 3T3 cells, the extracts were incubated with CaM kinase II. As assessed by DNA mobility shift analysis, this in vitro treatment led to the quantitative phosphorylation of wild-type SRF at Ser-103 (Fig. 6C).

We next compared the rates with which Ser-103-phosphorylated wild-type SRF and SRFm103 extracted from COS cells associated with and dissociated from the SRE. Western blot analysis confirmed that the two extracts being analyzed contained equal concentrations of SRF (Fig. 6D). SRF that is not phosphorylated at Ser-103 but is still stoichiometrically phosphorylated at the CKII site (SRFm103) was found to bind to the SRE more slowly than SRF that is phosphorylated at both the Ser-103 and CKII sites (SRF wt; Fig. 7A and B). In contrast, Ser-103 phosphorylation has little effect on the rate with which SRF dissociates from the SRE (Fig. 7C). To determine the effect of Ser-103 phosphorylation on the rate of SRF dissociation from the SRE, SRF-SRE binding reactions that had reached equilibrium were incubated with a large excess of unlabeled SRE for various amounts of time, and the amount of SRF that remained complexed to the SRE was analyzed by a DNA mobility shift assay. Thus, even when SRF is phosphorylated at the CKII site, Ser-103 phosphorylation increases the rate with which SRF binds to the SRE but does not affect its rate of dissociation. We conclude therefore that phosphorylation at Ser-103 increases the equilibrium binding affinity of SRF for the SRE. These observations suggest that upon serum stimulation of 3T3 cells, Ser-103 phosphorylation of SRF enhances the association of SRF with the SREs within the regulatory regions of IEGs and thereby plays a role in triggering IEG activation.

pp90^{rsk} has numerous features expected of the kinase that phosphorylates SRF upon growth factor stimulation. To characterize the signaling pathway(s) that mediates the growth factor-induced phosphorylation of SRF and leads to IEG

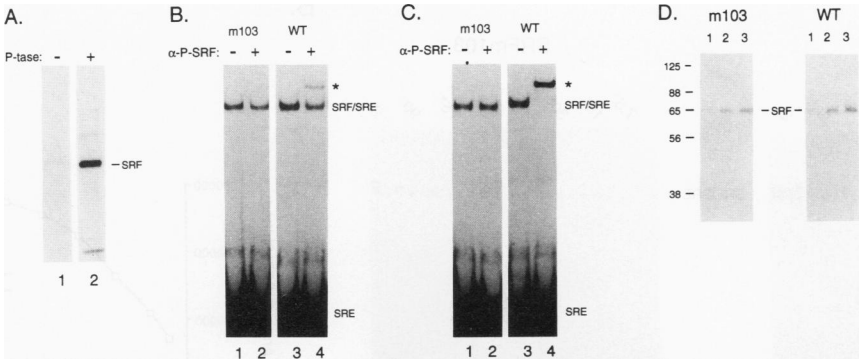

FIG. 6. Analysis of the phosphorylation state of SRF overexpressed in COS cells. (A) Nuclear extracts of COS cells transfected with wild-type SRF were treated with calf intestinal alkaline phosphatase (P-tase) (+) or mock treated (−). SRF was then immunoprecipitated from the extracts with anti-N-SRF antibodies and incubated with purified CKII in the presence of ATP. The band corresponding to SRF is marked. (B and C) DNA mobility shift analysis to detect the presence of SRF phosphorylated at Ser-103. Nuclear extracts from COS cells transfected with wild-type SRF (WT) or SRFm103 (m103) that were untreated (B) or incubated with CaM kinase II to phosphorylate wild-type SRF at Ser-103 (C) were analyzed by mobility shift to detect the presence of SRF phosphorylated at Ser-103. Extracts were incubated with ^{32}P-labeled SRE in the presence of BSA (−) or anti-P-SRF antibodies (α-P-SRF) (+), as indicated. Positions of the free probe (SRE) and the SRF-bound probe (SRF/SRE) are indicated. Each asterisk marks the position of the SRF-SRE complex whose migration is further retarded by interaction with anti-P-SRF antibodies. (D) Nuclear extracts from COS cells transfected with wild-type SRF or SRFm103 that were incubated with CaM kinase II (analyzed in panel C) were immunoblotted with anti-N-SRF antibodies; 1, 2, and 3 μl of extract were analyzed. The band corresponding to SRF is marked. Sizes are indicated in kilodaltons.

activation, various protein kinases were tested for the ability to phosphorylate SRF in vitro. The protein kinase(s) that catalyzes the growth factor-induced phosphorylation of SRF in vivo would be expected to have several properties. First, the enzyme must be a Ser/Thr kinase, since SRF is inducibly phosphorylated on Ser-103. Second, the SRF kinase must be localized, at least in part, to the nucleus, since previous studies indicate that SRF is localized exclusively to the nucleus, both before and after growth factor stimulation (25, 54). Third, the kinase activity must be growth factor inducible, with kinetics that parallel the rapid phosphorylation of SRF observed in vivo (Fig. 1C and 2). Kinases that fulfill these criteria include the ribosomal S6 kinases pp90^{rsk} and pp70-S6K (9a, 11, 21) and the MAP kinase pp44^{mapk} (11, 15). SRF was found to be efficiently phosphorylated in vitro by p90^{rsk} (see below). In contrast, neither pp44^{mapk} nor pp70-S6K was able to phosphorylate SRF, although these kinases were capable of phosphorylating previously characterized substrates (Fig. 8).

The ability of pp90^{rsk} to phosphorylate SRF was first demonstrated by using activated enzyme that was immunoprecipitated from serum-stimulated 3T3 cells with specific anti-pp90^{rsk} antibodies (Fig. 9A). An immune complex kinase assay in which SRF substrate was added to a washed anti-pp90^{rsk} immunoprecipitate in the presence of ATP was performed. Previous studies have shown that pp90^{rsk} immunoprecipitated from quiescent fibroblasts is inactive in an immune complex kinase assay. However, within 5 min of growth factor addition, pp90^{rsk} is activated, as measured by its ability to phosphorylate ribosomal protein S6 (9, 10, 71). We found that pp90^{rsk} phosphorylates SRF in an immune complex kinase assay and that its ability to phosphorylate SRF is growth factor inducible. pp90^{rsk} immunoprecipitated from cells stimulated with serum for 10 min, but not from quiescent cells, phosphorylates SRF effectively (Fig. 9A, lanes 1 and 2). In contrast, preimmune serum fails to immunoprecipitate an SRF kinase from serum-starved or serum-stimulated cells (data not shown).

To rule out the possibility that the anti-pp90^{rsk} antibodies were immunoprecipitating pp90^{rsk} in a complex with another growth factor-inducible kinase that was actually the SRF kinase, several additional experiments were performed. We demonstrated that both partially purified pp90^{rsk} (Fig. 9B) and baculovirus-expressed pp90^{rsk} (Fig. 9C) were also capable of effectively phosphorylating SRF. Taken together, these in vitro kinase experiments suggest that SRF is a direct substrate of pp90^{rsk}.

Subcellular fractionation of total cell lysates prior to immunoprecipitation revealed that pp90^{rsk} present in the nuclear as well as the cytoplasmic fraction of serum-stimulated 3T3 cells is capable of phosphorylating SRF (Fig. 9A, lanes 3 to 10). The kinetics of nuclear pp90^{rsk} activation in serum-stimulated cells directly parallel the kinetics of SRF phosphorylation and c-*fos* transcriptional activation (compare Fig. 9A and 1C) (33). pp90^{rsk} from cells stimulated with serum for 5 min phosphorylates SRF very efficiently. Its ability to phosphorylate SRF is still enhanced 15 min after stimulation before decreasing toward basal levels at 60 min. Taken together, these results suggest that the nuclear form of pp90^{rsk} is activated in its ability to phosphorylate SRF with kinetics that parallel the induced phosphorylation of SRF observed in vivo.

In considering further the possibility that pp90^{rsk} mediates the growth factor-induced phosphorylation of SRF, we tested whether pp90^{rsk} phosphorylates SRF at the same site, Ser-103, that is inducibly modified in vivo upon growth factor stimulation. Phosphorylation of SRF by pp90^{rsk} in vitro was found to generate a phosphotryptic peptide that comigrates, by two-dimensional thin-layer chromatography, with the SRF tryptic peptide A that becomes phosphorylated upon growth factor stimulation of 3T3 cells (Fig. 10A). The site of pp90^{rsk} phosphorylation within this peptide was shown to be Ser-103 by demonstrating that mutation of Ser-103 to alanine blocks the phosphorylation of SRF by pp90^{rsk} (Fig. 10B).

Taken together, these results demonstrate that pp90^{rsk} can directly phosphorylate SRF in vitro at the same site that is inducibly modified in vivo. Furthermore, pp90^{rsk} that is localized to the nucleus is activated in its ability to phosphorylate SRF with the appropriate kinetics expected of the

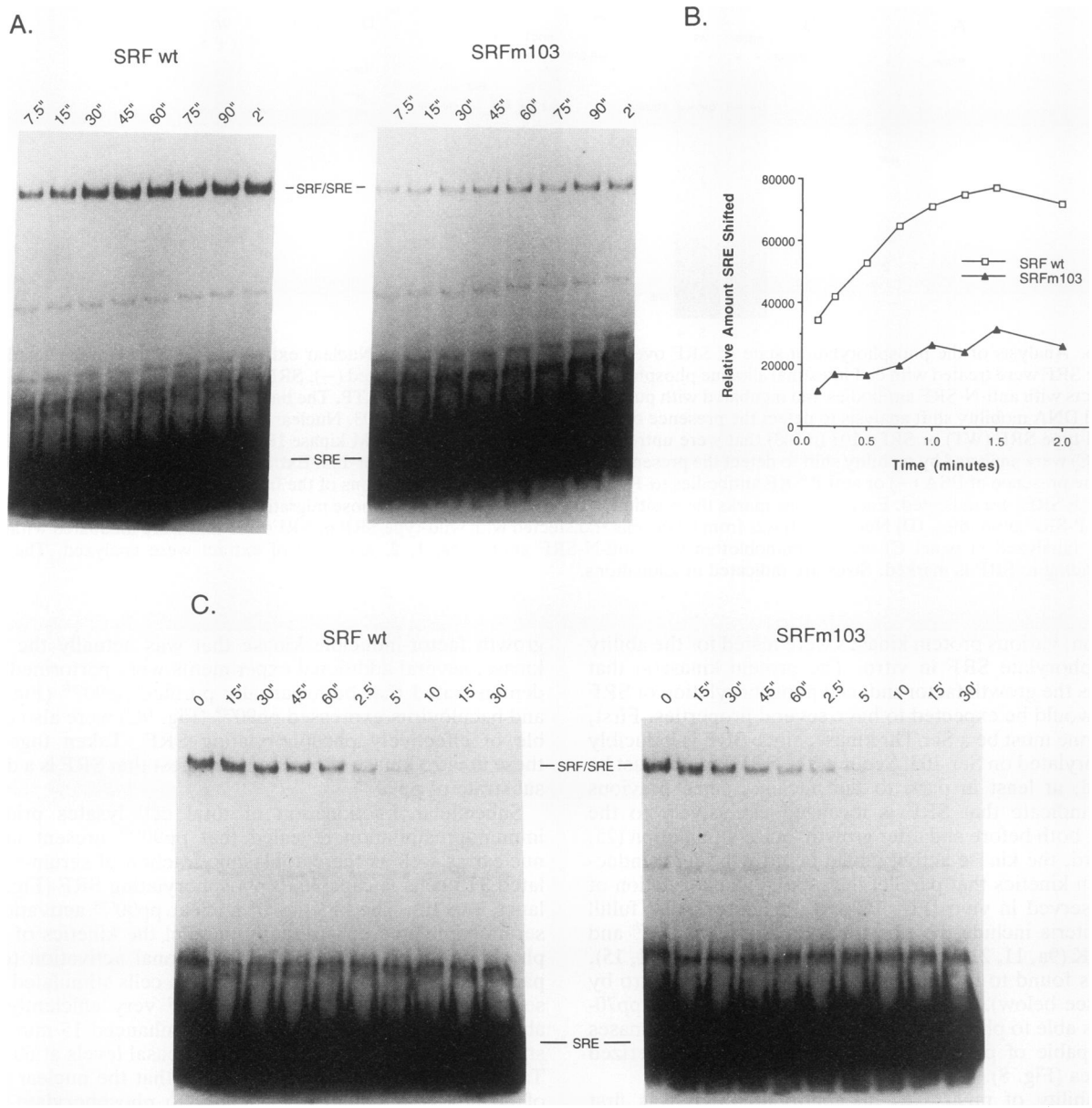

FIG. 7. Ser-103 phosphorylation of SRF increases the rate and affinity with which it binds to the SRE. (A) DNA mobility shift analysis to measure the rates with which Ser-103-phosphorylated wild-type SRF (SRF wt) and SRFm103 bind to the SRE. Extracts containing equivalent amounts of the two forms of SRF (Fig. 6D) were incubated with ^{32}P-labeled SRE for the indicated amounts of time (", seconds; ', minutes) before being loaded on the gel. (B) The results of the experiment shown in panel A were quantitated on a Molecular Dynamics PhosphorImager and expressed in relative units as the amount of ^{32}P-labeled SRE probe that was bound to the SRE as a function of time. (C) DNA mobility shift analysis to measure the rates with which Ser-103-phosphorylated wild-type SRF and SRFm103 dissociate from the SRE. Binding reaction mixtures were incubated with a 150-fold excess of unlabeled SRE for the indicated amounts of time. Positions of the free probe (SRE) and the SRF-bound probe (SRF/SRE) are indicated.

kinase that phosphorylates SRF. Thus, pp90^{rsk} is a strong candidate for being the enzyme that catalyzes the growth factor-induced modification of SRF.

DISCUSSION

To begin to elucidate the mechanism by which a growth factor-induced signal is transduced to the nucleus to activate IEG transcription, the phosphorylation state of SRF was examined before and after stimulation of quiescent 3T3 fibroblasts. SRF was found to be inducibly phosphorylated at Ser-103 within minutes of stimulation with serum or purified growth factors. The kinetics of Ser-103 phosphorylation and dephosphorylation parallel the kinetics of induction and repression of transcription of c-*fos* and other IEGs (2, 33). Furthermore, phosphorylation of SRF at Ser-103 was found to increase the rate and affinity with which SRF binds to the SRE. Given that SRF-binding sites are found upstream of a number of IEGs (12, 43, 60) and that the interaction of SRF with the SRE appears to be essential for

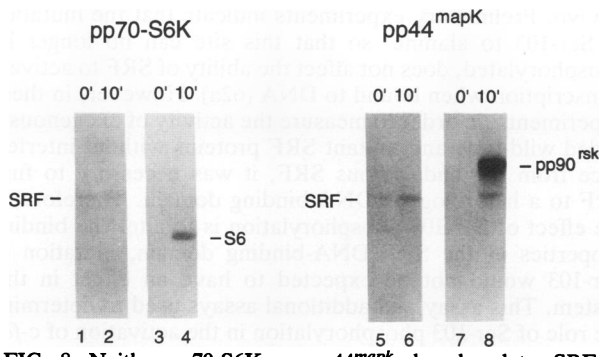

FIG. 8. Neither pp70-S6K nor pp44^{mapk} phosphorylates SRF in vitro. pp70-S6K (lanes 1 to 4) and pp44^{mapk} (lanes 5 to 8) were immunoprecipitated from quiescent (0′) or 10-min serum-stimulated (10′) cells and used in an immune complex kinase assay with bSRF (lanes 1, 2, 5, and 6), S6 (lanes 3 and 4), and pp90^{rsk} (lanes 7 and 8) as substrates. It has previously been shown that pp70-S6K and pp44^{mapk} from serum-stimulated but not quiescent cells efficiently phosphorylate the ribosomal S6 protein (9) and pp90^{rsk} (13), respectively. The low level of SRF phosphorylation (lanes 1, 2, 5, and 6) is most likely due to a contaminating activity in the immunoprecipitates and not to phosphorylation of SRF by pp70-S6K or pp44^{mapk}, since the phosphorylation of SRF in these reactions is not serum inducible.

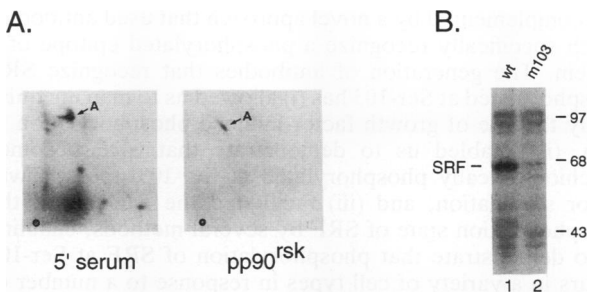

FIG. 10. pp90^{rsk} phosphorylates SRF at Ser-103. (A) Phosphotryptic peptide maps of SRF immunoprecipitated from ^{32}P-labeled Swiss 3T3 cells that were stimulated with serum for 5 min (5′ serum) and of bacterially expressed mouse SRF (56a) phosphorylated in vitro by pp90^{rsk} (pp90^{rsk}). In mixing experiments, the growth factor-induced peptide marked A was found to comigrate with the SRF peptide A phosphorylated by pp90^{rsk} in vitro (data not shown). (B) pp90^{rsk} immunoprecipitated from 5-min serum-stimulated HeLa cells was incubated with lysates from bacteria that overexpress full-length wild-type SRF (wt) or SRFm103 (m103). Equivalent results were obtained for pp90^{rsk} immunoprecipitated from Swiss 3T3 cells. Sizes are indicated in kilodaltons.

the induction and repression of c-*fos* transcription in response to serum (63), these results suggest that the serum-induced phosphorylation of SRF plays an important role in the activation of IEG transcription.

The change in SRF phosphorylation that we detect within minutes of growth factor stimulation has not been observed previously. However, several aspects of our experimental approach for analyzing SRF phosphorylation differ from the approach used by others (40, 49, 58) and could account for our ability to detect a growth factor-regulated modification of SRF. In our experiments, ^{32}P-labeled cellular extracts were generated by a rapid and highly denaturing lysis procedure that should inactivate phosphatases and kinases that might otherwise alter the SRF phosphorylation pattern during the preparation of the extract for immunoprecipitation. In addition, we developed conditions for labeling SRF with ^{32}P that did not adventitiously lead to activation, prior to serum stimulation, of the signaling pathway(s) that mediates c-*fos* induction. This was accomplished by monitoring, in parallel with the analysis of SRF phosphorylation, both activation of the growth factor-induced kinase pp90^{rsk} and stimulation of c-*fos* protein synthesis.

The traditional approach in which phosphopeptide maps are used to analyze the phosphorylation state of a protein

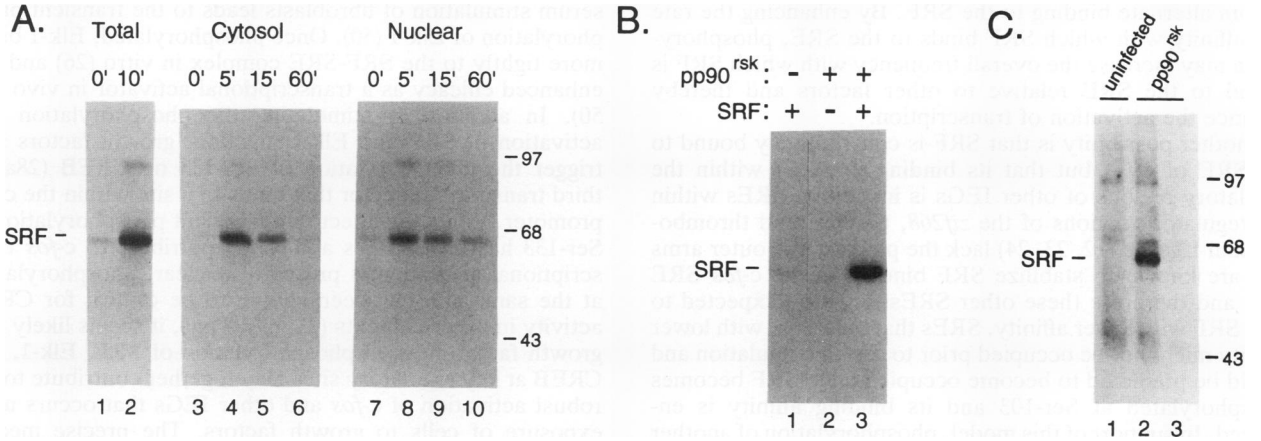

FIG. 9. SRF is a substrate for pp90^{rsk}. (A) An immune complex kinase assay was performed in which bvSRF was added to pp90^{rsk} immunoprecipitated from quiescent cells or cells stimulated with serum for the indicated amounts of time (minutes). In lanes 1 and 2, pp90^{rsk} was immunoprecipitated from total cell lysates; in lanes 3 to 10, pp90^{rsk} was immunoprecipitated from lysates that were first separated into cytoplasmic (lanes 3 to 6) and nuclear (lanes 7 to 10) fractions. Under these separation conditions, it has previously been shown that cytoplasmic and nuclear markers are found in the appropriate fractions (11). Sizes are indicated in kilodaltons. (B) bvSRF and pp90^{rsk} that was partially purified from serum-stimulated cells were incubated either alone or together, as indicated, in the presence of ATP under the conditions described in Materials and Methods. The position of the band corresponding to SRF is indicated. (C) Extracts from uninfected Sf9 cells or cells infected with baculovirus-containing pp90^{rsk} were immunoprecipitated with anti-pp90^{rsk} antibodies (lanes 1 and 2) or preimmune serum (lane 3). Each immune complex was incubated with SRF (TrpE-SRF-N) in the presence of ATP under conditions described in Materials and Methods. Sizes are indicated in kilodaltons.

was complemented by a novel approach that used antibodies which specifically recognize a phosphorylated epitope of a protein. The generation of antibodies that recognize SRF phosphorylated at Ser-103 has (i) allowed us to map unequivocally the site of growth factor-induced phosphorylation in vivo, (ii) enabled us to demonstrate that SRF becomes stoichiometrically phosphorylated at Ser-103 upon growth factor stimulation, and (iii) facilitated the analysis of the phosphorylation state of SRF by several methods, enabling us to demonstrate that phosphorylation of SRF at Ser-103 occurs in a variety of cell types in response to a number of different extracellular stimuli.

Our finding that SRF becomes transiently phosphorylated upon growth factor stimulation suggested that the role that SRF plays in regulating IEG transcription needed to be examined more closely. Therefore, various mechanisms by which Ser-103 phosphorylation might enhance the ability of SRF to stimulate c-*fos* transcription were investigated. We have found that Ser-103 phosphorylation causes a significant enhancement in the rate with which SRF associates with the c-*fos* SRE and increases SRF's overall affinity for the SRE severalfold. Janknecht et al. (40) also found that mutation of Ser-103 decreased the binding affinity of SRF overexpressed in vaccinia virus-infected HeLa cells. We conclude that a change in the affinity of SRF for SREs within the regulatory regions of IEGs seems likely to occur in vivo upon growth factor stimulation, since the phosphorylation of Ser-103 significantly enhances the affinity of binding of SRF to the SRE even when SRF is phosphorylated at additional sites (e.g., the CKII site) as it is in vivo.

How can we reconcile the suggestion that growth factor stimulation leads to the enhanced binding of SRF to the SRE with the previous demonstration, by in vivo genomic footprinting (36, 42), that the c-*fos* SRE is occupied before, during, and after serum stimulation of 3T3 fibroblasts? One possibility that cannot be excluded by the genomic footprinting experiments is that there is an exchange of factors at the c-*fos* SRE such that SRF, once phosphorylated, displaces a repressor that is bound to the SRE prior to stimulation. Alternatively, a dynamic state may exist in which multiple factors alternate binding to the SRE. By enhancing the rate and affinity with which SRF binds to the SRE, phosphorylation may increase the overall frequency with which SRF is bound to the SRE relative to other factors and thereby enhance the activation of transcription.

Another possibility is that SRF is constitutively bound to the SRE of c-*fos* but that its binding to SREs within the regulatory regions of other IEGs is inducible. SREs within the regulatory regions of the *zif268*, β-actin, and thrombospondin 1 genes (12, 23, 24) lack the palindromic outer arms that are known to stabilize SRF binding to the c-*fos* SRE (63), and therefore these other SREs might be expected to bind SRF with lower affinity. SREs that bind SRF with lower affinity might not be occupied prior to serum stimulation and would be predicted to become occupied once SRF becomes phosphorylated at Ser-103 and its binding affinity is enhanced. In support of this model, phosphorylation of another transcription factor, the cyclic AMP response element-binding protein (CREB), was recently shown to specifically enhance its binding to weak sites in vivo, while its association with higher-affinity sites was apparently unaffected (55).

Although we have shown that Ser-103 phosphorylation increases the affinity of SRF for the SRE and is closely correlated with the induction of c-*fos* transcription, it remains to be demonstrated that Ser-103 phosphorylation of SRF plays a direct role in the activation of c-*fos* transcription in vivo. Preliminary experiments indicate that the mutation of Ser-103 to alanine, so that this site can no longer be phosphorylated, does not affect the ability of SRF to activate transcription when bound to DNA (62a). However, in these experiments, in order to measure the activity of exogenously added wild-type and mutant SRF proteins without interference from the endogenous SRF, it was necessary to fuse SRF to a heterologous DNA-binding domain. Therefore, if the effect of Ser-103 phosphorylation is to alter the binding properties of the SRF DNA-binding domain, mutation of Ser-103 would not be expected to have an effect in this system. This assay and additional assays used to determine the role of Ser-103 phosphorylation in the activation of c-*fos* transcription are also limited by their reliance on the transfection of large amounts of SRF effector and SRE-containing reporter constructs into cells. Such conditions are likely to obscure an effect that Ser-103 phosphorylation might have on the rate and affinity with which SRF binds to the SRE in vivo. Thus, novel approaches may need to be developed to show that SRF Ser-103 phosphorylation plays a role in the activation of c-*fos* transcription.

In addition to its clear effect on the affinity of SRF for the SRE, Ser-103 phosphorylation may also affect SRF function in other ways. Preliminary experiments indicate that the phosphorylation of Ser-103 does not affect the ability of SRF to interact with Elk-1 (53a). However, it remains possible that Ser-103 phosphorylation can affect the ability of SRF to interact with other factors involved in the activation of c-*fos* transcription. Interestingly, comparison of the *Xenopus* SRF genes (54a) with those of humans (56) and mice (56a) reveals that much of the amino terminus of *Xenopus* SRF has diverged significantly from the human and mouse SRF genes, resulting in the absence of an equivalent Ser-103 phosphorylation site. This finding raises the possibility that the *Xenopus* SRF gene has evolved an alternative mechanism to carry out the function that Ser-103 phosphorylation has in the human and mouse SRF proteins.

Growth factor-induced phosphorylation of SRF at Ser-103 may be only one of several mechanisms that have evolved to control c-*fos* transcription. A recent report suggests that serum stimulation of fibroblasts leads to the transient phosphorylation of Elk-1 (50). Once phosphorylated, Elk-1 binds more tightly to the SRF-SRE complex in vitro (26) and has enhanced efficacy as a transcriptional activator in vivo (38, 50). In addition to stimulating the phosphorylation and activation of SRF and Elk-1 function, growth factors also trigger the phosphorylation of Ser-133 on CREB (28a), a third transcription factor that binds to a site within the c-*fos* promoter. While the effect that transient phosphorylation of Ser-133 has on CREB's ability to contribute to c-*fos* transcriptional activation is presently unclear, phosphorylation at the same site has been shown to be critical for CREB activity in other contexts (29, 69). Thus, it seems likely that growth factor-induced phosphorylation of SRF, Elk-1, and CREB at key regulatory sites may together contribute to the robust activation of c-*fos* and other IEGs that occurs upon exposure of cells to growth factors. The precise mechanism(s) by which phosphorylation of these three factors, either independently or jointly, leads to the activation of c-*fos* transcription remains to be established.

To understand the pathway by which growth factor stimulation leads to the rapid phosphorylation of SRF, protein kinases that can catalyze this phosphorylation event were identified. Our results implicate the Ser/Thr kinase pp90^{rsk} as one kinase that can catalyze the growth factor-induced phosphorylation of SRF in vivo. First, pp90^{rsk} phosphory-

lates SRF on Ser-103, the same site that is inducibly phosphorylated in vivo. Second, the kinetics of activation of the nuclear form of pp90^{rsk} in serum-stimulated cells, with respect to its ability to phosphorylate SRF in vitro, directly parallel the kinetics of SRF phosphorylation in vivo. Third, previous studies demonstrate a strong correlation between the activation of pp90^{rsk} and the activation of c-*fos* transcription in that all of the extracellular stimuli that are known to activate pp90^{rsk} (10, 71) also lead to the rapid activation of c-*fos* transcription through the SRE (reviewed in reference 76).

Although pp90^{rsk} was initially characterized on the basis of its ability to phosphorylate ribosomal protein S6 in extracts obtained from growth factor-stimulated cells (reviewed in reference 21), several recent studies indicate that this is not likely to be its actual function and that the in vivo targets of pp90^{rsk} remain to be identified (5, 14). The experiments described here have led to the characterization of SRF as a probable in vivo substrate of pp90^{rsk} and have provided the first evidence that pp90^{rsk} may be directly involved in the regulation of transcription of c-*fos* and other IEGs.

In addition to pp90^{rsk}, we have identified a Ca^{2+}/calmodulin-dependent kinase, CaM kinase II, that effectively phosphorylates SRF Ser-103 in vitro (53b). However, unlike pp90^{rsk}, whose in vivo activation by growth factors was found to correlate with SRF Ser-103 phosphorylation, CaM kinase II activation in vivo does not always coincide with growth factor induction of SRF Ser-103 phosphorylation (53b). Therefore, we favor the idea that in 3T3 cells, pp90^{rsk}, rather than CaM kinase II, mediates the growth factor-induced phosphorylation of SRF. We suspect that in other cell types, in response to different extracellular stimuli, CaM kinase II and possibly other kinases may catalyze the phosphorylation of SRF at Ser-103 and regulate its activity.

The finding that pp90^{rsk} may mediate the growth factor-induced phosphorylation of SRF allows us to propose a pathway by which a growth factor-induced signal is transduced from the plasma membrane to the nucleus. In this pathway, growth factor binding activates the receptor tyrosine kinase, which, through two or more intermediate factors, leads to the Tyr/Thr phosphorylation of the MAP kinases (reviewed in references 5 and 15). Recent studies have implicated Ras as one of the intermediate factors, since a dominant-negative mutant of Ras can block growth factor induction of MAP kinase activity (18, 72, 77, 80). In addition, a factor that can activate the MAP kinases has been purified from growth factor-stimulated cells, and its gene has been cloned (reviewed in reference 1). MAP kinase, once activated, phosphorylates and activates pp90^{rsk} (13, 70). We propose that activated pp90^{rsk} then phosphorylates SRF in the nucleus at Ser-103, thus enhancing its ability to bind to the SRE and thereby activating c-*fos* transcription.

A similar growth factor-inducible signaling pathway has been suggested to mediate the phosphorylation of Elk-1 that occurs upon serum stimulation of 3T3 cells (26, 50). The growth factor-regulated MAP kinase has been shown to phosphorylate Elk-1 in vitro at sites that regulate its ability to activate transcription in vivo (38, 50). Taken together, these findings suggest that growth factor stimulation of the MAP kinase/pp90^{rsk} signaling pathway results in the modification of multiple components of an SRE-bound complex and leads to the rapid and robust activation of c-*fos* transcription. Direct proof of the involvement of MAP kinase and pp90^{rsk} in the activation of c-*fos* transcription must await the development of assays that will allow us to assess the effect that in vivo perturbation or enhancement of the activity of the MAP and pp90^{rsk} kinases has on c-*fos* expression.

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