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The Serum Response Factor Is Extensively Modified by Phosphorylation Following Its Synthesis in Serum-Stimulated Fibroblasts

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Growth factor regulation of c-*fos* proto-oncogene transcription is mediated by a 20-bp region of dyad symmetry, termed the serum response element. The inner core of this element binds a 67-kDa phosphoprotein, the serum response factor (SRF), that is thought to play a pivotal role in the c-*fos* transcriptional response. To investigate the mechanism by which SRF regulates c-*fos* expression, we generated polyclonal anti-SRF antibodies and used these antibodies to analyze the biochemical properties of SRF. These studies indicate that the synthesis of SRF is transient, occurring within 30 min to 4 h after serum stimulation of quiescent fibroblasts. Newly synthesized SRF is transported to the nucleus, where it is increasingly modified by phosphorylation during progression through the cell cycle. Within 2 h of serum stimulation, differentially modified forms of SRF can be distinguished on the basis of the ability to bind a synthetic serum response element. SRF protein exhibits a half-life of greater than 12 h and is predominantly nuclear, with no change occurring in its localization upon serum stimulation. We find that the induction of SRF synthesis is regulated at the transcriptional level and that cytoplasmic SRF mRNA is transiently expressed with somewhat delayed kinetics compared with c-*fos* mRNA expression. These features of SRF expression suggest a model whereby newly synthesized SRF functions in the shutoff of c-*fos* transcription.

The c-*fos* proto-oncogene is a member of a family of immediate-early genes (IEGs) that are transiently activated as an early response to growth factor stimulation (1, 26, 29, 51). A variety of experiments suggest that c-*fos* plays a critical role in the response of cells to growth factors and that aberrant production of Fos protein can lead to oncogenesis (11, 22, 32). Elucidation of the biochemical mechanisms controlling c-*fos* transcription is thus likely to give important insights into the mechanisms by which growth factor-regulated gene expression controls proliferation in normal and transformed cells.

c-*fos* mRNA is almost undetectable in quiescent fibroblasts; however, within minutes of exposure to purified growth factors or serum, there is a rapid increase in the level of c-*fos* transcription (19, 24, 33). This increase is independent of protein synthesis (7, 17, 24, 27, 33), suggesting that the growth factor-induced signal is transduced from the membrane to the nucleus via the modification of preexisting factors. c-*fos* transcriptional induction is transient, with transcription returning to the level present in unstimulated cells within 60 min of stimulation. In contrast to activation, transcriptional repression requires protein synthesis, suggesting the involvement of a labile or newly synthesized repressor. Several reports indicate that the Fos protein plays a role in the repression of its own transcription (15, 23, 30, 43, 44).

Mutational analyses have defined a 20-bp region of dyad symmetry, located 300 bp upstream from the start site of c-*fos* mRNA synthesis, as the major determinant of the transcriptional response to purified growth factors and serum (12, 13, 18, 53). This sequence, termed the serum response element (SRE), is sufficient to confer rapid and transient transcriptional kinetics on minimal c-*fos* or heterologous promoters (23, 41), suggesting that the SRE is a key site of regulation for both transcriptional activation and repression (reviewed in reference 40). The importance of the SRE for growth factor-regulated transcription is further supported by its presence within the regulatory region of at least four other transiently expressed IEGs (reviewed in reference 56).

A nuclear protein, the serum response factor (SRF), that binds specifically to the c-*fos* SRE has been identified (14, 18, 38, 54). The ability of SRF to interact with mutant SRE elements in vitro directly correlates with the ability of the SRE to confer serum responsiveness in vivo (13, 18, 28, 49, 55). This finding suggests that SRF is an important regulator of c-*fos* transcription.

Several proteins other than SRF have been identified as interacting with the SRE (42, 48, 58). One of these proteins, p62/ternary complex factor (p62/TCF), interacts with SRF when it is bound to the SRE to form a ternary complex. Although p62/TCF does not bind to the SRE on its own, it enhances the interaction of SRF with the SRE (45). In vivo analyses of subtle mutations in the SRE that do not affect the interaction of SRF with the SRE but that impair the ability of p62/TCF to form the ternary complex reveal the existence of p62/TCF-dependent and p62/TCF-independent pathways (16). The p62/TCF-dependent pathway appears to be utilized by growth factors that activate protein kinase C (16).

Functional analysis of SRF has been facilitated by its purification (39, 46, 55) and by cloning of the human SRF gene (34). SRF is a 62- to 67-kDa protein that binds the SRE as a dimer (34). When assayed in vitro, purified SRF enhances the level of RNA polymerase II transcription in an SRE-dependent manner (34, 37). Additional experiments indicate that SRF is modified posttranslationally by phosphorylation (36) and that in vitro phosphorylation by casein kinase II (CKII) enhances SRF's association with the SRE in

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vitro (31). The importance of this phosphorylation event for SRF function in vivo remains to be determined.

Although these studies suggest that SRF is likely to be of central importance in mediating the c-*fos* transcriptional response to growth factors, the specific role played by SRF in this response remains unclear. To investigate the function of SRF in growth factor-stimulated cells and to identify SRF-associated proteins that may play a role in this response, we generated anti-SRF antibodies capable of precipitating the SRF protein. In this report, we describe these antibodies and the results of experiments characterizing the in vivo state of SRF in serum-stimulated NIH 3T3 fibroblasts.

MATERIALS AND METHODS

Cell culture. Mouse NIH 3T3 cells were grown in 10% CO_2 in Dulbecco modified Eagle medium (DMEM; GIBCO) containing 10% heat-inactivated calf serum, 0.01% penicillin, and 0.01% streptomycin. Cells were made quiescent by growing them to 70 to 80% confluence and then replacing the growth medium with DMEM containing 0.5% calf serum for 24 to 40 h. Cultures were serum stimulated by replacing the starvation medium with DMEM containing 20% heat-inactivated fetal calf serum. All sera were purchased from Sigma Chemical Co.

Preparation and affinity purification of anti-SRF antibodies. To generate the TrpE–SRF-N fusion protein, a 597-bp *Sma*I-*Bgl*II fragment (from a partial *Sma*I digest) of the human SRF cDNA clone pT7ΔATG (34) was inserted into a pATH10 vector (2). This region of SRF encodes nearly all of the amino-terminal half of the SRF protein (amino acids 46 to 245), which includes the DNA-binding and dimerization domains. The TrpE–SRF-C fusion protein was generated by cloning a 792-bp *Bgl*II fragment containing the carboxy-terminal half of the SRF protein (amino acids 245 to 508) into pATH10. Bacteria transformed with either the TrpE–SRF-N or -C construct were induced to express the TrpE-SRF fusion proteins by addition of indoleacrylic acid to the culture medium (2). The fusion proteins were purified and used to produce polyclonal antisera as previously described (57).

Antibodies were affinity purified by a modification of a previously published procedure (57). Briefly, TrpE antibodies were removed by passing the crude serum over a column containing TrpE protein coupled to Affi-Gel 10 beads (Bio-Rad). The flowthrough from this column was then passed over an Affi-Gel 10 column containing the TrpE-SRF fusion protein. Antibodies specific for SRF were then eluted from the TrpE-SRF column with 0.1 M glycine (pH 2.5) and collected in tubes containing bovine serum albumin and an amount of Tris-HCl (pH 8.8) calculated to raise the pH to 7.0.

Anti-Fos antibodies were generated and purified as previously described (57).

Cell labeling, lysis, and immunoprecipitation. [^{35}S]methionine-labeled cells were prepared as follows. Serum-starved cells were incubated for 30 min in methionine-free, serum-free DMEM (GIBCO) and then either labeled by the addition of 0.5 mCi of [^{35}S]methionine per ml or stimulated and labeled by replacing the medium with methionine-free DMEM containing 20% dialyzed fetal calf serum and 0.5 mCi of [^{35}S]methionine (Trans-Label; ICN) per ml for the indicated times. ^{32}P-labeled cells were prepared by starving confluent cells in phosphate-free DMEM (ICN) containing 0.5% dialyzed fetal calf serum for 24 h and then adding 1.5 mCi of $^{32}P_i$ (ICN) per ml directly to the medium for 3 h.

To prepare 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 one of two methods. Nondenatured extracts were prepared by lysing cells on ice in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, and 1 μg of pepstatin A per ml. Denatured extracts were prepared by adding a boiling solution of SDS buffer (0.5% SDS, 50 mM Tris-HCl [pH 7.4]) to the cells, boiling the lysate for 5 min, and then diluting the lysate by the addition of 4 volumes of 1.25× RIPA buffer without SDS.

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-SRF antibodies as described previously (57). Nonspecific and specific competitions were performed by preincubating the anti-SRF antibodies with TrpE and TrpE-SRF proteins, respectively. Following fractionation of the immunoprecipitated proteins by SDS-polyacrylamide gel electrophoresis (PAGE), fluorography was performed on ^{35}S-labeled proteins by incubating the gel in 1 M sodium salicylate for 30 min.

Phosphatase treatment of immunoprecipitated SRF. Immunoprecipitations were carried out as described above except that following the last RIPA wash, the protein A-Sepharose beads with bound immune complexes were washed twice in RIPA buffer without SDS or deoxycholate and twice in 0.1 M morpholineethanesulfonic acid (MES; pH 6.0). Samples were then incubated with 2 U of potato acid phosphatase (Boehringer Mannheim Biochemicals) at 37°C for 30 min in the presence or absence of phosphatase inhibitors (0.2 M sodium pyrophosphate, 0.2 mM sodium orthovanadate, and 50 mM sodium fluoride). The immune complexes were then washed once with 0.1 M MES (pH 6.0) containing 0.2 M sodium phosphate (pH 6.5) before being loaded onto an SDS-polyacrylamide gel.

Column chromatography of cell lysates. To affinity purify ^{35}S-labeled SRF prior to immunoprecipitation, 10^7 serum-stimulated 3T3 cells were stimulated and labeled with [^{35}S]methionine for 90 min, and RIPA extracts were prepared as described above. Labeled extracts were diluted with buffer D (9), containing 0.45 M KCl, to a final concentration of 0.3 M KCl; 6 μg of poly(dI-dC), 4 μg of pUC19, and 50 μl of DSE affinity resin (55) were then added. After incubation for 2 h at 4°C with tumbling, the unbound fraction was collected and the SRE affinity resin was washed with buffer D containing successively higher concentrations of KCl. Each KCl wash was collected and adjusted to RIPA lysis buffer conditions containing a final KCl concentration of 150 mM. SRF was identified in the flowthrough and KCl wash fractions by immunoprecipitation with anti-SRF antibody as described above.

Northern (RNA) analysis. The ^{32}P-labeled probe used to detect SRF mRNA was synthesized by random-primed oligonucleotide labeling of a 597-bp *Sma*I-*Bgl*II fragment from the amino-terminal end of the human SRF clone pT7ΔATG (34). Cytoplasmic RNA was isolated and Northern analysis was performed as previously described (4) except that following hybridization, SRF Northern blots were washed two times at 65°C in 2× SSC–0.1% SDS (1×

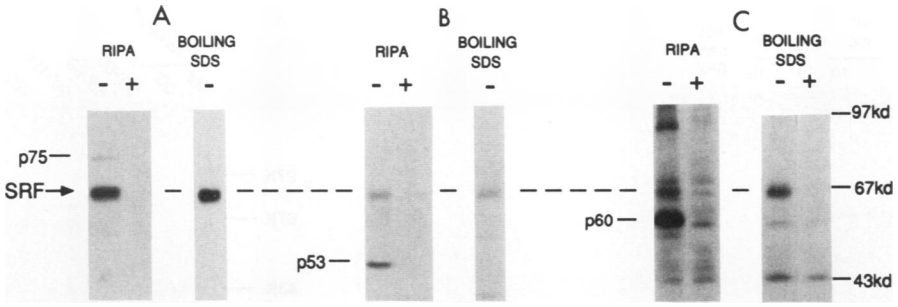

FIG. 1. Characterization of anti-SRF antibodies. RIPA or boiling SDS lysates were prepared from serum-starved NIH 3T3 cells that were serum stimulated for 2 h in [^{35}S]methionine containing medium as described in Materials and Methods. SRF was immunoprecipitated from both types of lysates, using antibodies raised against the N-terminal half of SRF (antibody 1 [A] or antibody 2 [B]) or the C-terminal half of SRF (C). Each antibody was preincubated with either a TrpE-nonspecific competitor (−) or the appropriate specific competitor (TrpE-SRF; +), as indicated. All three antibodies immunoprecipitate SRF from cell lysates prepared by either RIPA or boiling SDS lysis. Also indicated are proteins which coimmunoprecipitate with SRF when cells are lysed under RIPA but not boiling SDS conditions (p75, p53, and p60).

SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and three times at 65°C in 0.5× SSC–0.1% SDS.

Nuclear run-on transcription. Direct analysis of the level of transcription of various genes was performed as previously described (41). The linearized SRF clone pT7ΔATG was used to detect SRF transcripts.

Subcellular fractionation. Metabolically labeled cells were separated into nuclear and cytoplasmic fractions as described by Curran et al. (8). Lactate dehydrogenase activity was monitored as a cytoplasmic marker and was assayed by the method of Cabaud and Wroblewski (5). Reagents were purchased from Sigma, and the assay was carried out as described by the manufacturer.

Immunohistochemistry. Cells were grown on coverslips, and immunostaining was performed as described by Curran et al. (8).

Western immunoblot analysis. HeLa nuclear extracts were prepared by the method of Dignam et al. (9) and fractionated over BioRex-70 (Bio-Rad) and DNA-cellulose (Sigma) columns before being passed over an SRE affinity column as described by Treisman (55). Fractions were electrophoresed by SDS-PAGE, transferred to nitrocellulose (52), and immunoblotted with anti-SRF-N1 antibodies. Bound antibody was detected by using goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Promega) and visualized as described by the supplier.

RESULTS

Characterization of SRF by using anti-SRF antibodies. To obtain large quantities of purified protein for the preparation of anti-SRF antibodies, SRF was expressed at high levels in bacteria. A DNA fragment that encodes 200 amino acids from the N-terminal half of SRF, including the DNA-binding and dimerization domain, was cloned into a TrpE bacterial expression vector so that this region of SRF was expressed as a TrpE-SRF fusion protein. The DNA fragment coding for the 264 C-terminal amino acids of SRF was also cloned into the TrpE vector to generate a second TrpE-SRF fusion protein. Both the N- and C-terminal SRF fusion proteins (TrpE–SRF-N and -C) were expressed at high levels in *Escherichia coli* upon induction of the tryptophan operon (data not shown). After purification, these proteins were used to generate two antisera that recognize the N-terminal half of SRF (anti-SRF-N1 and -N2) and one antiserum that recognizes the C-terminal half of SRF (anti-SRF-C).

The anti-SRF antibodies immunoprecipitate a diffusely migrating protein in the range of 63 to 65 kDa from [^{35}S]methionine-labeled extracts of serum-stimulated 3T3 fibroblasts (Fig. 1). This protein is not immunoprecipitated by preimmune sera (data not shown) or if the anti-SRF antibodies are preincubated with the appropriate TrpE-SRF fusion protein prior to immunoprecipitation (Fig. 1). The 63- to 65-kDa protein migrates on SDS-PAGE similarly to purified human SRF, suggesting that the anti-SRF antibodies are immunoprecipitating murine SRF. This was confirmed by [^{35}S]methionine tryptic mapping, which demonstrated that the immunoprecipitated 63- to 65-kDa protein is highly related to in vitro-transcribed and -translated human SRF (data not shown). The 63- to 65-kDa murine SRF is also functionally similar to human SRF in that it interacts specifically with the SRE. As described below, passage of radiolabeled 3T3 cell extracts over an SRE affinity column prior to immunoprecipitation revealed that a large proportion of the 63- to 65-kDa protein binds tightly to the column in the presence of 0.3 M KCl and is eluted when the KCl concentration is raised to 1.0 M.

The anti-SRF-N1 antibody was also found to specifically immunoprecipitate in vitro-transcribed and -translated human SRF (Fig. 2) or human SRF from radiolabeled HeLa cell extracts (data not shown). The specificity of the anti-SRF-N1 antibody was confirmed further by Western blotting. As shown in Fig. 2, the anti-SRF-N1 antibody recognizes a 67-kDa protein present in HeLa nuclear extracts that comigrates with HeLa SRF purified by passage over an SRE affinity column. Taken together, these results indicate that the anti-SRF-N1 antibody specifically recognizes mouse and human SRF. A similar series of experiments established that the anti-SRF-N2 and anti-SRF-C antibodies also specifically immunoprecipitate SRF (data not shown).

When radiolabeled 3T3 cells are extracted under nondenaturing conditions and immunoprecipitated with anti-SRF-N1 or -N2 antibodies, two additional proteins (53 and 75 kDa) that coprecipitate with SRF are detected (Fig. 1). Detection of p53 and p75 is dependent on different subpopulations of antibodies in the polyclonal preparation, since antibodies affinity purified from sera obtained from two different rabbits vary in the ability to precipitate these proteins. The C-terminal anti-SRF antibodies do not immunoprecipitate p53 and p75, but instead immunoprecipitate a 60-kDa protein in addition to SRF. The immunoprecipitation of all three coprecipitating proteins appears specific inasmuch as it is blocked if the anti-SRF antibodies are preincu-

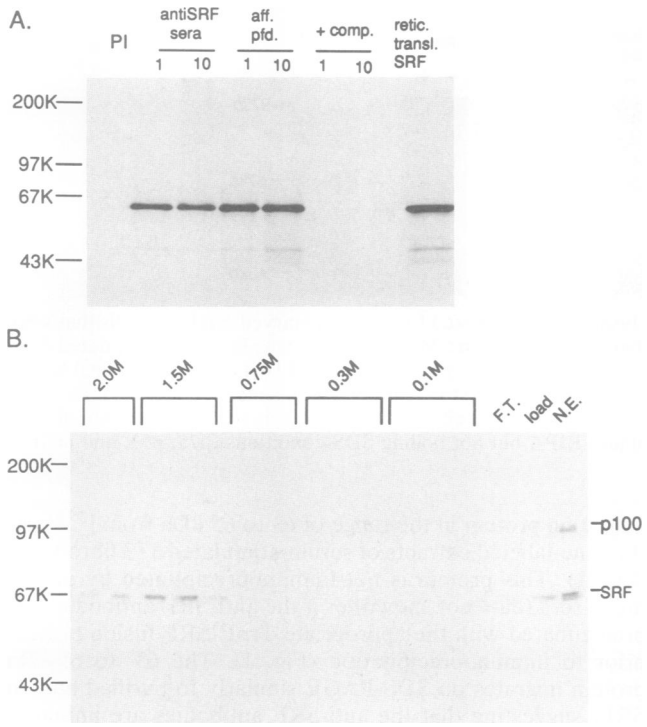

FIG. 2. Evidence that anti-SRF antibodies recognize in vitro translated SRF and SRF purified from HeLa cells. (A) Preimmune, crude, and affinity-purified anti-SRF-N1 antibodies were tested for the ability to immunoprecipitate ^{35}S-labeled SRF generated by in vitro transcription and translation from the human SRF clone pT7ΔATG (34). The amount of ^{35}S-labeled SRF shown in the rightmost lane was tested for its ability to be immunoprecipitated by 10 μl of preimmune sera (PI) and 1 or 10 μl of the following: crude anti-SRF-N1 (antiSRF sera), affinity-purified anti-SRF-N1 (aff. pfd.), and affinity-purified anti-SRF-N1 that had been preincubated with the TrpE–SRF-N bacterial fusion protein (+ comp.). (B) SRF was partially purified by fractionation of a HeLa nuclear extract over BioRex-70 and DNA cellulose. The SRF-containing fraction from the DNA-cellulose column was applied to an SRE affinity column in buffer D (9) containing increasing concentrations of KCl. Portions of the HeLa nuclear extract (N.E.), the SRE affinity column load, flowthrough (F.T.), and increasing KCl elution fractions were immunoblotted by using the anti-SRF-N1 antibody. The nuclear extract contains two immunoreactive proteins of 67 and 100 kDa. The 67-kDa immunoreactive protein elutes at high salt from the SRE column, identifying this species as HeLa SRF (34). The identity of p100, which appears to elute from the SRE column at low KCl concentrations, is unknown.

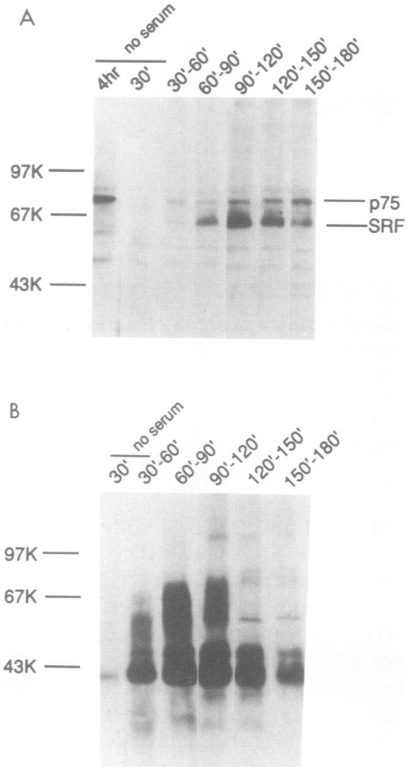

FIG. 3. Induction of SRF protein synthesis by serum stimulation. Serum-starved NIH 3T3 cells either were left unstimulated (no serum) and labeled with [^{35}S]methionine for 4 h or 30 min or were stimulated with 20% fetal calf serum for 60, 90, 120, 150, or 180 min (') and labeled with [^{35}S]methionine for the last 30 min of stimulation. RIPA cell lysates of each time point were then divided into two aliquots and immunoprecipitated with anti-SRF-N1 (A) or anti-Fos (B) antibodies. The positions of SRF and p75 are indicated. In the Fos immunoprecipitation, differentially modified Fos proteins make up the large smear between 55 and 70 kDa, while the 39- to 45-kDa bands most likely consist of Fos- and Jun-related proteins.

bated with the appropriate SRF fusion protein but not if the antibodies are preincubated with a nonspecific competitor.

It is not yet clear whether the SRF coprecipitating proteins interact with or share epitopes with SRF. If 3T3 cells are lysed by boiling in 0.5% SDS prior to addition of the anti-SRF antibodies, SRF, but none of the coprecipitating proteins, is immunoprecipitated (Fig. 1). This result is consistent with the possibility that the coprecipitating proteins interact directly with SRF.

Of particular interest is the 60-kDa protein, since this protein is similar in size to p62/TCF. The possibility that this protein is p62/TCF is supported by the finding that the 60-kDa protein is coprecipitated with the anti-SRF-C antibodies but not the anti-SRF-N antibodies. Since the interaction of p62/TCF with the SRF/SRE complex requires the DNA-binding/dimerization domain of SRF (48) to which the anti-SRF-N antibodies were raised, it might be expected that the N-terminal antibodies would disrupt the p62/TCF-SRF interaction or might not recognize SRF when it is bound to p62/TCF.

Murine SRF is transiently synthesized after serum stimulation. In contrast to the results obtained with serum-stimulated 3T3 cells (Fig. 1), we were unable to detect SRF in [^{35}S]methionine-labeled extracts prepared from continuously growing (data not shown) or serum-starved (Fig. 3) 3T3 fibroblasts. This result is surprising since SRF-binding activity is easily detected in these extracts (13, 14; unpublished results). A possible explanation for our inability to detect SRF by biosynthetic labeling of serum-starved cells is that SRF is a stable protein that is actively synthesized only in growth factor-treated cells. To test this possibility, serum-starved cells were pulse-labeled with [^{35}S]methionine before stimulation and at various times after serum stimulation, and SRF was immunoprecipitated with anti-SRF antibodies. This experiment revealed that SRF is not actively synthesized in serum-starved fibroblasts but is newly synthesized upon serum stimulation, with kinetics that are delayed relative to Fos induction (Fig. 3). SRF synthesis is first detected approximately 30 to 60 min after serum addition

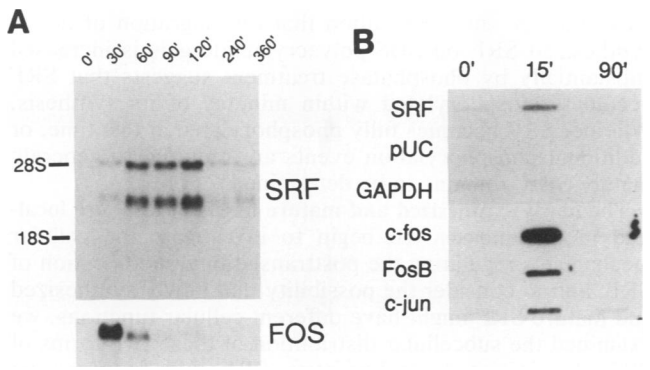

FIG. 4. Transient induction of SRF mRNA synthesis upon serum stimulation. (A) Cytoplasmic RNA from quiescent NIH 3T3 cells that were stimulated for the specified times (indicated in minutes) were assayed by Northern blot analysis. The filter was hybridized with a probe specific for SRF and then stripped and hybridized with a probe specific for c-*fos*. (B) Nuclear run-on transcription analysis was performed on nuclei isolated from quiescent NIH 3T3 cells that were serum stimulated for the specified times (indicated in minutes). The lack of hybridization of ^{32}P-labeled RNA to pUC sequences indicates that the signal detected by the other gene-containing plasmids is due to hybridization with the insert and not the vector sequences. The relatively constant level of transcription of the gluteraldehyde 3-phosphate dehydrogenase (GAPDH) gene demonstrates that approximately equal amounts of ^{32}P-labeled RNA were hybridized to the filters. The transcription levels of several other IEGs (c-*fos*, *fosB*, and c-*jun*) are shown for comparison with level of the SRF gene.

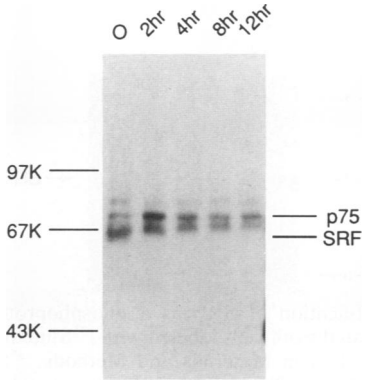

FIG. 5. Evidence that newly synthesized SRF is stable and is extensively modified during the first few hours following serum stimulation. Quiescent NIH 3T3 cells were pulse-labeled for 2 h by incubation in medium containing 20% dialyzed fetal calf serum and [^{35}S]methionine and then chased for the indicated times in stimulation medium containing unlabeled methionine. Lysates of these cells were then immunoprecipitated with anti-SRF-N1 antibodies. The positions of SRF and p75 are shown.

and peaks by 90 to 150 min (Fig. 3). Within 6 h of the initial stimulation event, SRF synthesis returns nearly to the level present in unstimulated cells (data not shown). SRF synthesis is also stimulated when starved 3T3 cells are exposed to the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate and when the pheochromocytoma cell line PC12 is treated with nerve growth factor (34a), suggesting that the induction of SRF synthesis is a general cellular response to treatment with growth factors.

Serum induces SRF protein synthesis by stimulating transcription of the SRF gene. By nuclear run-on transcription analysis, SRF transcription is barely detected in uninduced cells but is induced severalfold as early as 15 min after serum stimulation of quiescent 3T3 cells (Fig. 4B). Induction of SRF transcription is transient, returning to basal levels by 90 min after serum stimulation. Northern analysis of cytoplasmic mRNA isolated at various times after stimulation reveals two mRNA species of approximately 2.9 and 4.5 kb that hybridize to the human SRF gene probe and that are inducible by serum (Fig. 4A). SRF mRNA species of similar size have been detected in HeLa cells and have been shown to be induced fivefold upon serum stimulation (34). These two mRNAs were shown to have identical coding region sequences but different-length 3′ untranslated regions (34).

The expression pattern of SRF mRNAs parallels the synthesis profile of the SRF protein. Overall, these mRNAs display many of the characteristics of IEGs. The SRF mRNAs are difficult to detect in the absence of serum but are transiently induced approximately 15-fold within 2 h of serum stimulation (Fig. 4A). In addition, SRF mRNA synthesis is superinduced in the presence of inhibitors of protein synthesis such as cycloheximide (data not shown; 34). The accumulation of SRF mRNA is delayed relative to that of c-*fos* mRNA (Fig. 4A). Of interest is the finding that the SRF protein level peaks when the c-*fos* mRNA level is declining (Fig. 3 and 4A). This finding is consistent with the possibility that newly synthesized SRF is involved in the regulation of the shutoff of c-*fos* transcription.

SRF is relatively stable and is extensively modified during the first few hours following serum stimulation. Unlike many IEG-encoded proteins, which are very labile, newly synthesized SRF is a stable protein. SRF was immunoprecipitated from quiescent 3T3 cells that had been serum stimulated and labeled with [^{35}S]methionine for 2 h and then chased for various periods of time between 0 and 12 h with medium containing unlabeled methionine (Fig. 5). By this pulse-chase analysis, SRF was found to have a half-life of approximately 12 h. In contrast, the half-lives of two other IEG-encoded proteins, Fos and Nur77, are approximately 120 and 30 min, respectively (10, 20, 57).

Pulse-chase analysis also revealed that newly synthesized SRF undergoes a series of successive posttranslational modifications that retard its migration on SDS-polyacrylamide gels (Fig. 5). While newly synthesized SRF migrates as a 63- to 65-kDa species, it is modified in a number of discrete steps during the next 12 h until it is converted to a 67-kDa mature form. Several findings suggest that the posttranslational modifications of SRF are due in large part to the phosphorylation of SRF at multiple sites (see below).

Immunoprecipitation of SRF from extracts of ^{32}P-labeled 3T3 cells, prepared in the presence of SDS and phosphatase inhibitors, demonstrates that murine SRF is a phosphoprotein (Fig. 6; see also reference 36). As shown in Fig. 6, anti-SRF antibodies specifically immunoprecipitate a 67-kDa protein from ^{32}P-labeled cells which comigrates with SRF that is immunoprecipitated from [^{35}S]methionine-labeled cells. Phosphotryptic mapping of immunoprecipitated SRF demonstrates that this protein is phosphorylated at a minimum of four sites (data not shown).

To examine the possibility that the newly synthesized and mature forms of SRF migrated differently on SDS-gels because of differences in their state of phosphorylation, we determined the effect that phosphatase treatment had on the migration of each of these forms of SRF. The 63- to 65-kDa newly synthesized form of SRF was immunoprecipitated from quiescent 3T3 cells that had been serum stimulated and

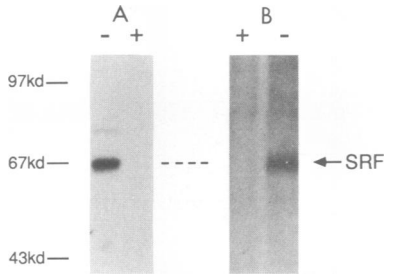

FIG. 6. Identification of SRF as a phosphoprotein. SRF was immunoprecipitated from cells labeled with [^{35}S]methionine (A) and ^{32}P (B) as described in Materials and Methods. ^{35}S-labeled cells were lysed in RIPA buffer; ^{32}P-labeled cells were lysed in boiling SDS. Immunoprecipitations were performed by using anti-SRF-N1 antibodies in the presence (+) or absence (−) of competitor TrpE–SRF-N.

labeled with [^{35}S]methionine for 90 min. The 67-kDa mature form of SRF was immunoprecipitated from cells that had been similarly pulse-labeled for 90 min but then chased for 12 h in medium containing unlabeled methionine.

Figure 7 shows that treatment of the SRF immunoprecipitates with potato acid phosphatase increases the mobility of both the newly synthesized and mature forms of SRF, suggesting that both forms of SRF are modified by phosphorylation. However, after phosphatase treatment, mature SRF still migrates more slowly than newly synthesized SRF, indicating that at least one of the posttranslational modifications that converts newly synthesized SRF to the mature form is a phosphatase-resistant modification. A previous study has shown that SRF is glycosylated (45), raising the possibility that the newly synthesized SRF is converted to mature SRF at least in part by a glycosylation event.

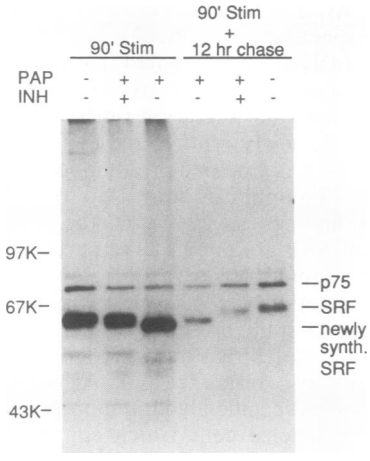

FIG. 7. Phosphatase treatment of newly synthesized and mature forms of SRF. The newly synthesized (90′ [90-min] Stim) and mature (90′ Stim + 12 hr chase) forms of SRF were immunoprecipitated from RIPA lysates as described in the text. Following immunoprecipitation with anti-SRF-N1 antibodies, each immunoprecipitate was then split three ways and either left untreated, incubated with potato acid phosphatase (PAP) plus inhibitors (INH), or incubated with potato acid phosphatase alone, as indicated. Positions of the untreated newly synthesized SRF (newly synth. SRF) and the untreated mature SRF (SRF) are marked. The position of p75, whose migration is unaltered by PAP treatment, is also shown.

Nevertheless, the observation that the migration of newly synthesized SRF on SDS-polyacrylamide gels is increased substantially by phosphatase treatment suggests that SRF becomes phosphorylated within minutes of its synthesis. Whether SRF becomes fully phosphorylated at this time, or additional phosphorylation events are required to generate mature SRF, remains to be determined.

The newly synthesized and mature forms of SRF are localized in the nucleus. To begin to investigate the cellular mechanisms regulating the posttranslational modification of SRF, and to consider the possibility that newly synthesized and mature SRF might have different cellular functions, we examined the subcellular distribution of these two forms of SRF. By immunohistochemistry, SRF was found to be localized almost exclusively in the nucleus (Fig. 8A to D). When serum-starved 3T3 cells were left untreated or exposed to serum for 10 or 90 min and then processed for immunostaining with anti-SRF-N2 antibodies, intense nuclear immunoreactivity was detected in all cases. Anti-SRF antibody staining of the nucleus is specific inasmuch as it is completely blocked by preincubation of the antibody with the TrpE–SRF-N fusion protein but not by preincubation with the TrpE protein (Fig. 8A to D). The immunolocalization of SRF in the nucleus was corroborated by using the anti-SRF-C antibodies (data not shown).

We considered the possibilities that particular forms of SRF might be relatively minor components of the overall SRF population and that their subcellular localization might be difficult to discern in the immunohistochemical experiments described above. Therefore, to examine further the subcellular localization of different forms of SRF, newly synthesized and mature SRF were labeled with [^{35}S]methionine as described above, and their distribution was examined by using a subcellular fractionation protocol (8). Separation of nuclei and cytoplasm revealed that both newly synthesized and mature SRF are localized exclusively in the nuclear fraction. The integrity of the nuclear and cytoplasmic fractions was established by demonstrating that another nuclear protein, Fos, was localized in the nuclear fraction (Fig. 8E) and that the cytoplasmic enzyme lactate dehydrogenase was present almost exclusively in the cytoplasmic fraction (data not shown). We conclude that within minutes of its synthesis, SRF is translocated to the nucleus. Since the most recently synthesized and least modified form of SRF is localized in the nucleus, this finding suggests that the posttranslational modifications of SRF occur primarily in the nucleus.

Newly synthesized SRF consists of multiple forms that differ in their ability to bind to an SRE. To establish the importance of posttranslational modifications for SRF function, differentially modified forms of SRF were tested for the ability to interact with an SRE. The newly synthesized and mature forms of SRF were labeled with [^{35}S]methionine as described above, and cellular extracts were passed over an SRE affinity column. After collection of the unbound fraction, the SRE affinity column was washed with buffer containing increasing concentrations of KCl. Column fractions were then immunoprecipitated with anti-SRF antibodies. This analysis revealed that newly synthesized SRF is composed of at least two distinct forms of SRF: (i) a 63- to 64-kDa form found in the flowthrough that does not bind the SRE and (ii) a 65-kDa form that binds the SRE, eluting from the column in 0.75 to 1.25 M KCl (Fig. 9). These two species of SRF appear to be present in approximately equal amounts in 3T3 cells pulse-labeled with [^{35}S]methionine for the first 90 min after stimulation. Whether the 63- to 64-kDa and 65-kDa

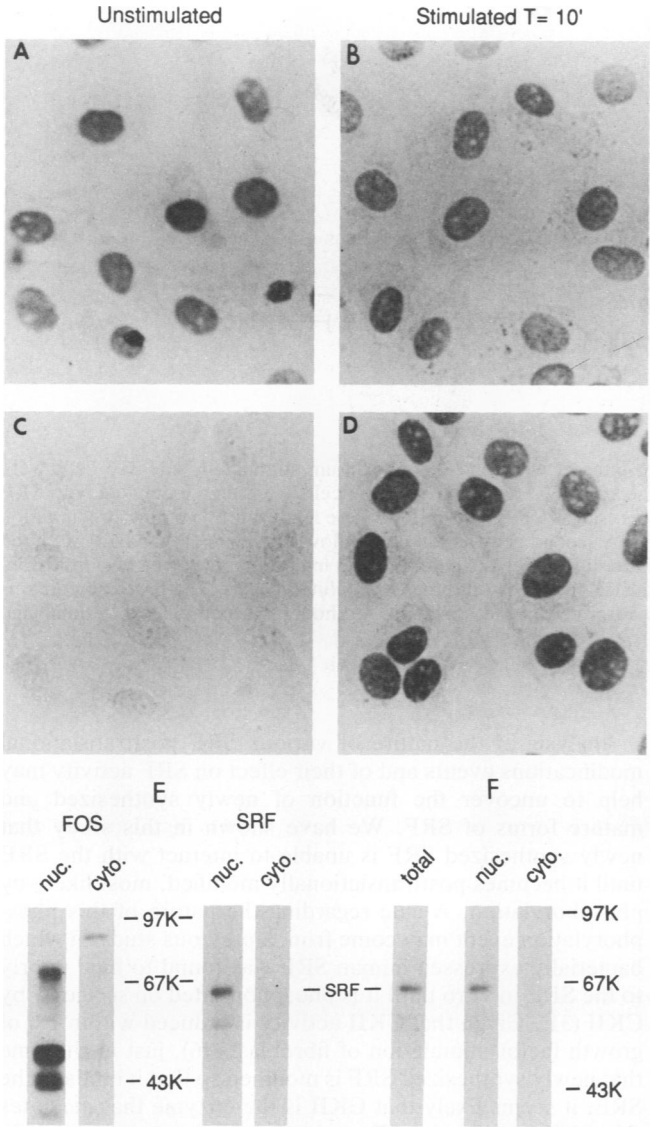

FIG. 8. Localization of SRF in the nucleus. (A to D) NIH 3T3 cells that were either unstimulated (A) or stimulated with 20% fetal calf serum for 10 (B) or 90 (D) min were fixed, permeabilized, and stained with anti-SRF-N2 antibodies preincubated with the TrpE protein or anti-SRF-N2 antibodies (preincubated with the TrpE–SRF-N protein) as competitor (C). (E and F) Analysis of SRF subcellular localization by cell fractionation. In panel E, quiescent NIH 3T3 cells were serum stimulated, labeled with [^{35}S]methionine for 2 h, and then separated, after hypotonic lysis, into nuclear (nuc.) and cytoplasmic (cyto.) fractions. The nuclear and cytoplasmic fractions were lysed in boiling SDS and immunoprecipitated with anti-SRF-N2 or anti-Fos antibodies. The position of SRF in the nuclear fraction is marked. The c-Fos (55 to 70 kDa) and Fos- and Jun-related proteins (39 to 45 kDa) are found in the nuclear fraction. Greater than 95% of the total lactate dehydrogenase activity was found in the cytoplasmic fraction (data not shown). In panel F, quiescent NIH 3T3 cells were serum stimulated, labeled with [^{35}S]methionine for 3 h, chased for 15 h with medium containing unlabeled methionine, and then separated into nuclear (nuc.) and cytoplasmic (cyto.) fractions. The position of SRF in the total and nuclear fractions is shown. Greater than 90% of the total lactate dehydrogenase activity was found in the cytoplasmic fraction (data not shown).

forms of newly synthesized SRF differ in the extent of phosphorylation or in some other way remains to be determined. A strong possibility is that immediately after its synthesis, SRF is translocated to the nucleus as a 63- to 64-kDa protein that is incapable of binding to the SRE. Phosphorylation of this species may give rise to the 65-kDa form of SRF that is then capable of interacting with the SRE. As shown above, the 65-kDa form of SRF is further modified over time to generate the mature, 67-kDa form of SRF. Analysis of mature SRF's ability to bind an SRE affinity column indicates that this form of SRF is composed predominantly of species that bind tightly to the column and are eluted only when the KCl concentration is raised to greater than 0.75 M (Fig. 9B; data not shown). It is unclear if the newly synthesized 65-kDa form SRF and mature SRF differ with respect to affinity for the SRE.

DISCUSSION

Using specific anti-SRF antibodies, we have characterized the properties of SRF in serum-stimulated fibroblasts. We demonstrate that SRF is a 63- to 67-kDa phosphoprotein whose synthesis is activated within minutes of serum stimulation. Newly synthesized SRF is transported to the nucleus, where it undergoes a series of posttranslational alterations. An initial modification event, which is believed to be due to phosphorylation, converts SRF from a form that does not appreciably interact with the SRE to a form that binds tightly to the SRE. Subsequently, SRF becomes further modified over time, although the effect of these later posttranslational modifications on SRF function remains to be determined. Immunohistochemistry and subcellular fraction studies indicate that the variously modified forms of SRF are localized in the nucleus. This suggests that unlike other transcription factors that have been implicated in the process of signal transduction (e.g., NF-κB and heat shock factor [3, 25, 50]), the regulation of SRF activity occurs primarily in the nucleus and not in the cytoplasm.

Several recent studies suggest that SRF functions in the nucleus as part of a protein complex that includes p62/TCF (21, 45, 47, 48). The SRF complex is believed to mediate the activation of c-*fos* transcription that occurs upon serum stimulation. Our observation that several additional proteins (p53, p60, and p75) are coprecipitated with SRF in anti-SRF antibody immunoprecipitates is consistent with the idea that SRF regulates c-*fos* transcription in conjunction with other proteins. Whether the SRF coprecipitating proteins are SRF-related or SRF-interacting proteins and whether they play a critical role in the c-*fos* transcriptional response must await further experimentation.

The availability of anti-SRF antibodies that are capable of immunoprecipitating SRF from denatured cellular extracts will facilitate further investigation of the specific role that SRF plays in the c-*fos* transcriptional response. A reasonable hypothesis is that growth factors induce a rapid posttranslational modification of SRF that stimulates its ability to induce c-*fos* transcription. While one previous study suggested that SRF is not modified posttranslationally within the time needed to activate c-*fos* transcription (36), the possibility that phosphorylation of SRF or its associated proteins is important for c-*fos* activation has not been thoroughly investigated. The anti-SRF antibodies described in this report should allow the characterization of SRF's phosphorylation state in a variety of cell types, before and minutes after growth factor stimulation, under extraction conditions that are likely to preserve the in vivo pattern of phosphorylation.

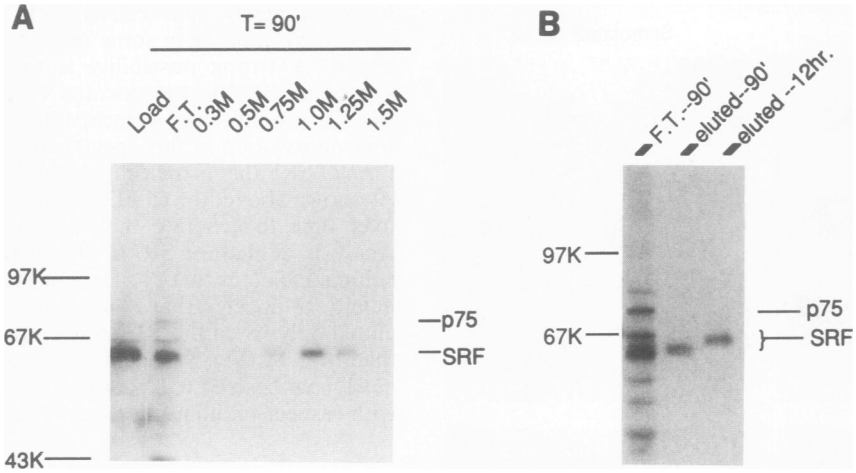

FIG. 9. Differentially modified forms of SRF vary in their abilities to interact with an SRE. (A) Serum-stimulated NIH 3T3 cells were pulse-labeled with [^{35}S]methionine for 90 min (90′) to label newly synthesized SRF. Extracts from these cells were then incubated with SRE affinity resin, and the flowthrough (F.T.) and elutions from washes of increasing KCl concentrations were analyzed by immunoprecipitation with the anti-SRF-N1 antibody as described in Materials and Methods. (B) A second gel was run on the flowthrough (F.T.) and 1.0 M eluted fractions from the experiment shown in panel A to better visualize size differences. Note the differences in migration of the newly synthesized SRF that does not bind the SRE (F.T.--90′) and the newly synthesized SRF that does bind the SRE (eluted--90′). The last lane contains mature SRF (immunoprecipitated from lysates of cells pulse-labeled as described in Materials and Methods and then chased in unlabeled stimulation medium for 12 h).

In addition to its postulated role in c-*fos* transcriptional activation, SRF may also function in the repression of transcription that occurs subsequent to the activation event. The observation reported here, that SRF is newly synthesized in growth factor-stimulated cells just at the time that c-*fos* transcription begins to decline, raises the interesting possibility that newly synthesized SRF functions as a repressor of c-*fos* transcription. Consistent with this idea are two additional findings: (i) an SRF-binding site confers both growth factor activation and repression of transcription to a heterologous gene (15, 23, 41) and (ii) repression of c-*fos* transcription is dependent on new protein synthesis (17).

To function effectively as a repressor, newly synthesized SRF would have to differ in some way from preexisting SRF that is present in the activation complex. We speculate that the posttranslational modifications of newly synthesized SRF that occur gradually after its transport to the nucleus may alter its activity. Once SRF is modified so that it can interact with the SRE, newly synthesized SRF may displace the preexisting activated form of SRF, thereby leading to repression. Alternatively, newly synthesized SRF may indirectly effect c-*fos* down-regulation by stimulating a repressor activity or sequestering an activator. To examine the importance of newly synthesized SRF in the shutoff of c-*fos* transcription, we are currently attempting to block growth factor-mediated synthesis of SRF by using SRF antisense mRNA.

Newly synthesized SRF may have other functions as well. One possibility is that it is a regulator of genes that are activated or repressed at later times after growth factor stimulation and thus may play a role in late G_1 and S phases of the cell cycle. The similarity in structure of SRF and the yeast protein MCM1, whose mutation leads to a defect in the maintenance of copy number of yeast minichromosomes (34, 35), raises the possibility that SRF may also function, either directly or indirectly, as a regulator of DNA replication. Further experimentation will be necessary to distinguish among these possibilities.

Analysis of the nature of various SRF posttranslational modifications events and of their effect on SRF activity may help to uncover the function of newly synthesized and mature forms of SRF. We have shown in this study that newly synthesized SRF is unable to interact with the SRE until it becomes posttranslationally modified, most likely by phosphorylation. A clue regarding the nature of this phosphorylation event may come from a previous study in which bacterially expressed human SRF was found to bind poorly to the SRE in vitro until it is phosphorylated on serine 85 by CKII (31). Given that CKII activity is induced within 1 h of growth factor stimulation of fibroblasts (6), just at the time that newly synthesized SRF is modified so that it binds to the SRE, it seems likely that CKII is the enzyme that mediates this SRF modification. Experiments are now under way in our laboratory to determine whether phosphorylation of newly synthesized SRF at serine 85 is the in vivo event that converts SRF to a form that is capable of interacting with the SRE. Additional studies will be required to identify the sites of modification that convert newly synthesized SRF to the mature form and to determine the importance of these modifications in regulating SRF's role in the activation and repression of c-*fos* transcription.

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