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Mutation of Serum Response Factor Phosphorylation Sites and the Mechanism by which Its DNA-Binding Activity Is Increased by Casein Kinase II

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Casein kinase II (CKII) phosphorylates the mammalian transcription factor serum response factor (SRF) on a serine residue(s) located within a region of the protein spanning amino acids 70 to 92, thereby enhancing its DNA-binding activity in vitro. We report here that serine 83 appears to be the residue phosphorylated by CKII but that three other serines in this region can also be involved in phosphorylation and the enhancement of DNA-binding activity. A mutant that contained glutamate residues in place of these serines had only low-level binding activity; however, when the serines were replaced with glutamates and further mutations were made that increased the negative charge of the region, the resulting mutant showed a constitutively high level of binding equal to that achieved by phosphorylation of wild-type SRF. We have investigated the mechanism by which phosphorylation of SRF increases its DNA-binding activity. We have ruled out the possibilities that phosphorylation affects SRF dimerization or relieves inhibition due to masking of the DNA-binding domain by an amino-terminal region of the protein. Rather, using partial proteolysis to probe SRF's structure, we find that the conformation of SRF's DNA-binding domain is altered by phosphorylation.

It has been well established that transcriptional activation of the proto-oncogene c-*fos* by serum and a variety of growth factors is mediated by a sequence element, called the serum response element (SRE), located at position −300 in relation to the transcription start site (3, 5, 8, 28, 29). This element binds a dimerized 64-kDa nuclear phosphoprotein termed the serum response factor (SRF) (6, 21, 29). SRF has recently been cloned and studied by using protein produced by in vitro translation (16). These studies localized the DNA-binding domain to a region in the center of the protein (amino acids 133 to 264) overlapping with the region required for dimerization (amino acids 168 to 222). Other studies demonstrated that SRF produced by in vitro translation, produced by *Escherichia coli*, or purified from HeLa cells was able to stimulate transcription in vitro, suggesting that SRF is directly involved in induction of c-*fos* expression (13, 16, 20).

Since serum induction of c-*fos* can occur in the presence of protein synthesis inhibitors (7), posttranslational events must be required to regulate c-*fos* expression. This can be achieved in a number of ways; two of the most likely are (i) modification of pre-existing SRF molecules that increases the transcriptional efficacy of the proteins and (ii) complexing of SRF molecules with regulatory proteins. We have shown that the DNA-binding activity of SRF is reduced by phosphatase treatment (19) and that the DNA-binding activity of SRF produced in bacteria is greatly enhanced when it is phosphorylated by casein kinase II (CKII) (13), a protein kinase whose activity is stimulated by growth factors (1, 2, 11, 25). Moreover, the site that is phosphorylated in vitro is also phosphorylated in vivo, demonstrating the physiological relevance of the event (13). Thus, it is possible that phosphorylation by CKII may be involved in the regulation of c-*fos* by growth factors.

In most cases, SRF's DNA-binding activity, when measured in nuclear extracts, has not been observed to change in response to serum or growth factor treatment of cells (3, 8, 23, 29). Only in epidermal growth factor-treated A431 cells has increased SRF binding been observed (21). It is possible, however, that SRF's DNA-binding activity changes during preparation of nuclear extracts (e.g., by phosphorylation or dephosphorylation). In vivo footprinting experiments in A431 cells (10) indicated that a factor(s) is constitutively bound to the SRE before and after epidermal growth factor treatment. This does not rule out, however, the possibility that differentially modified SRFs or distinct factors bind to the SRE in different cell states.

In order to further characterize SRF's phosphorylation, we have made missense mutations in putative phosphorylation sites. We have additionally analyzed how SRF phosphorylation at a site greater than 50 amino acids away from the DNA-binding domain might affect SRF's DNA-binding activity.

MATERIALS AND METHODS

Plasmid constructions. To construct the various missense mutants used in this study, a 1.6-kb *Xba*I fragment from pARSRF-Nde (13) containing the SRF coding region was subcloned into pTZ18U (14), a vector supplied by Bio-Rad for use in their Muta-Gene Phagemid In Vitro Mutagenesis Kit. After the desired mutations were created, the 1.6-kb *Xba*I fragment was subcloned back into the pAR3040 expression vector and used to transform bacteria from which protein extracts were prepared (26). A phosphorylated oligonucleotide, CGACTCGGAGGCCGGCGAGGAGG, was used to construct the Ala-85 mutant. In addition, the phosphorylated oligonucleotides CGAGGGCGACGCTGAGTC GGGCG, CGAGGGCGACGCTGAGGCCGGCG, GGGGG CCCTCTACGCCGGCCTCGAGGGCGACGCTG, and CG GAGTCGGGCGAGCTCGCGGAGCTGGGCGCCG were used to construct the Ala-83, Ala-83/85, AL 77-85, and LA 88/89 mutants, respectively. The AL 77-85 mutant was used

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as the template in constructing the Glu-X and Glu-83/85 mutants, using the phosphorylated oligonucleotides CTCTA CGAGGGCGAGGAGGGCGACGAGGAGGAGGGCGA GG and GCCTCGAGGGCGACGAGGAGGAGGGCGAGG AGGAGGAG, respectively. The Glu-83/85 mutant was used as the template in constructing the Glu-77-85 mutant, using the phosphorylated oligonucleotide GGGGCCCTCTACG AAGGCGAAGAGGGCGAC. Each of the mutations was sequenced to confirm the changes and in the case of Glu-X, to deduce the additional amino acid mutations.

The Δ1-114 mutant was constructed by digesting pARSRF-Nde with *Apa*I, blunting the ends with T4 DNA polymerase, ligating on *Bam*HI 10-mer phosphorylated linkers (New England BioLabs), digesting with *Bam*HI, gel purifying the 1.2-kb band, and subcloning this fragment into pAR3040 digested with *Bam*HI. Construction of the Δ339-508 mutant was as previously described (13).

Preparation of bacterial protein extracts. Bacterial protein extracts were prepared as previously described (13). Electroeluted bacterial SRF, used in the partial tryptic proteolysis experiments, was prepared as previously described (13) except that, rather than lyophilizing the eluate, the protein was precipitated with acetone as described elsewhere (9). The pellet was washed two times with 80% acetone, air dried at 37°C, and resuspended in BC100–6 M guanidine-HCl (BC100 is 20% glycerol, 100 mM KCl, 20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA). The solution was mixed for 40 min at room temperature to denature the protein and then dialyzed overnight against BC100 at 4°C to renature the protein. Insoluble material was removed by centrifugation for 5 min at 13,000 × *g*. The amounts of mutant proteins were quantitated by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels and further normalized by comparison in immunoblotting experiments, using an anti-SRF C-terminal peptide serum (13).

Gel mobility shift assay. For experiments demonstrating enhanced DNA binding of SRF proteins, the gel mobility shift assays were performed as described previously (13). About 0.5 ng of the various bacterially made SRF (BSRF) proteins contained in crude bacterial protein preparations was used per reaction. For the dimerization experiment, all samples were incubated at 37°C for 30 min in the same buffer used to phosphorylate SRF prior to incubating the protein with the gel mobility shift buffer (13) except that only the reactions involving phosphorylation contained CKII (2 μl of a phosphocellulose and MonoQ column-purified preparation [13]) and 400 μM ATP. To normalize for binding differences, about 20 times more unphosphorylated protein (see Fig. 5A, lanes 1 to 4) than phosphorylated protein (lanes 5 to 8) was used in the assay. Signals from the gels were quantitated, using a Phosphorimager and ImageQuant software (Molecular Dynamics).

Phosphorylation of SRF mutants. The various BSRF mutant proteins were phosphorylated as previously described (13) whereby 100 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear) and 20 μM nonradioactive ATP were used per point. After the incubation, SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (200 mM Tris-HCl [pH 6.8], 6% SDS, 30% glycerol, 15 mM EDTA, 1% beta-mercaptoethanol, 0.02% bromophenol blue) was added to the samples, which were boiled and then electrophoresed on an 8% SDS–polyacrylamide gel. Signals from the gel were quantitated by using a Phosphorimager (Molecular Dynamics).

Partial tryptic proteolysis of BSRF. For each point, about 100 ng of an electroeluted BSRF fusion protein in BC100 (containing the first 11 amino acids of the T7 gene 10 protein as well as 3 amino acids from a *Bam*HI linker and missing the first 9 amino acids of SRF [in BC100; 13]) was incubated for 55 min at 37°C in 25 μl of buffer containing 1 mM dithiothreitol, 3 mM $MgCl_2$, 40% BC100 (total including protein fractions), 0.05 μl of purified CKII (0.3 mg/ml, purified from bovine testes and kindly provided by E. Krebs; 27), with or without 400 μM ATP. The samples were then cooled for 5 min in a water bath at room temperature, and 1.25 μl of the indicated concentration of trypsin (porcine pancreas, excision grade; Calbiochem) was added to each sample, which was incubated at room temperature for 10 additional min. The reactions were stopped by addition of SDS-PAGE sample buffer and subsequent boiling for 5 min. The proteolysis products were electrophoresed on a 10% SDS–polyacrylamide gel and visualized by immunoblotting.

Immunoblotting was done as described previously (19) except that a 1:500 dilution of antiserum directed against a C-terminal synthetic peptide of SRF (13) was incubated with the nitrocellulose filter for 3 h at room temperature and subsequently incubated with the secondary antibody (anti-rabbit immunoglobulin G, alkaline phosphatase conjugated; Promega) for 30 min at room temperature.

RESULTS

Synthesis and analysis of mutant proteins. Missense mutations in the coding region of SRF were produced by using in vitro mutagenesis techniques. These mutations were placed into a bacterial expression vector, and mutant proteins were produced in *E. coli* as previously described (13). All proteins were quantitated by immunoblot analysis, using antiserum directed against an SRF C-terminal peptide. Figure 1 shows the various mutations that were made.

We suspected that serine 85 was the site of phosphorylation, since it is located next to a stretch of four acidic amino acids which is a consensus recognition sequence for CKII phosphorylation (12). Moreover, we previously found that deletion of amino acids 70 to 92 abolished phosphorylation and enhancement of DNA-binding activity (13). For these reasons, we decided to construct missense mutations in this region of the protein, changing the serine residues to either alanine or leucine, to see whether we could abolish phosphorylation and thus, presumably, the enhancement of DNA-binding activity. Either one, two, or four serines were mutated as indicated. Furthermore, we tried to abolish the CKII recognition site by changing two of the glutamate residues to leucine and alanine.

A gel mobility shift assay was used to quantitate the extent of DNA binding enhancement of the various mutants (Fig. 2). Crude bacterial lysates containing the mutated proteins were incubated with CKII with or without ATP, as indicated, subsequently incubated with a ^{32}P-labeled double-stranded oligonucleotide, XGL, containing a high-affinity SRF-binding site, and electrophoresed on a native polyacrylamide gel. We have previously found that ATP or GTP is required for phosphorylation and enhancement of DNA-binding activity such that incubations without ATP were performed to indicate the level of binding activity of the unphosphorylated protein (13). The quantitation of the level of increased DNA binding with versus without ATP is indicated above each pair of lanes. Surprisingly, a single mutation of serine 85 or 83 caused only a slight reduction in enhancement of DNA-binding activity (Fig. 2, cf. lanes 3 to 6 with lanes 1 and 2). Double mutation of these two serines caused a much greater reduction but did not abolish the

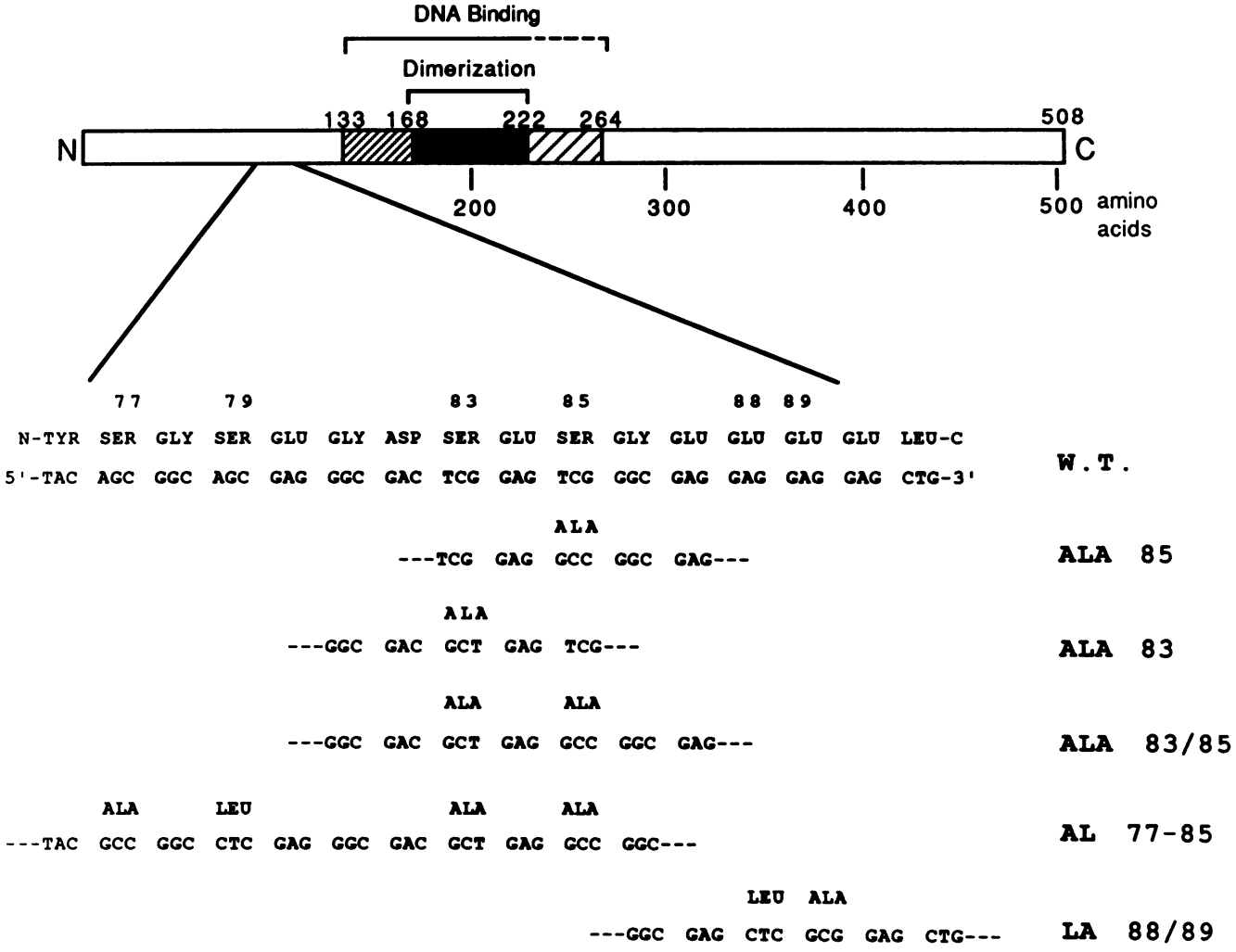

FIG. 1. SRF missense mutations. A schematic of SRF's domain structure (16) is shown (top) with the DNA-binding and dimerization domains indicated. Deletion of the region from amino acids 222 to 264 reduced DNA-binding activity but did not abolish it, while the other indicated domains were absolutely required for activity. Missense mutations were created in the region of amino acids 77 to 89 of SRF, using in vitro mutagenesis techniques. The exact protein and DNA sequence alterations in the various mutants are indicated. W.T., wild type.

effect completely (lanes 7 and 8). Mutations of all four serines at positions 77, 79, 83, and 85 did, however, completely abolish the ability of SRF's DNA-binding activity to be enhanced by CKII (lanes 9 and 10). This mutant, AL 77-85, exhibited binding activity comparable with that of a mutant with amino acids 70 to 92 deleted (data not shown).

We assayed the phosphorylation of these mutated proteins in order to compare it with the enhancement of DNA-binding activity. Phosphorylation by CKII in vitro was assayed by incubating the crude bacterial lysates containing the mutated SRF proteins with CKII and [γ-^{32}P]ATP followed by electrophoresis directly on an SDS-polyacrylamide gel (Fig. 3). It should be noted that the phosphorylation of SRF (lane 1) is quite specific, since a crude bacterial lysate in which SRF was not a major constituent (i.e., SRF was not a major band on a Coomassie blue-stained SDS-polyacrylamide gel) was used. Controls of bacterial lysate or CKII alone (lanes 8 and 9, respectively) showed no detectable phosphorylation in the region of SRF proteins. The effects of the mutations on SRF phosphorylation were similar but more drastic than the effects on DNA binding, as shown by the percent phosphorylation (relative to wild-type SRF) indicated above each lane. Again, of the single point mutations, mutation at serine 83 had the greatest effect, albeit small (lane 2), while mutation at all four serines was required to reduce phosphorylation to a level comparable with that of the Δ70-92 deletion mutant (lanes 5 and 7). The greater effects of the mutations on phosphorylation than on DNA binding suggest that a significant amount of binding enhancement can occur as a result of low levels of phosphorylation. The smaller effects on the DNA binding activities can be explained by the nonlinearity of the gel mobility shift assay (13; and unpublished data). Low, but detectable, phosphorylation was also observed for the AL 77-85 and Δ70-92 mutants (lanes 5 and 7). This suggests that CKII is phosphorylating these proteins nonspecifically somewhere other than the CKII site and that this low-level phosphorylation has no effect on binding.

We further tried to abolish phosphorylation and DNA-binding enhancement by disrupting the putative CKII recognition site in SRF. Since CKII recognizes serines followed

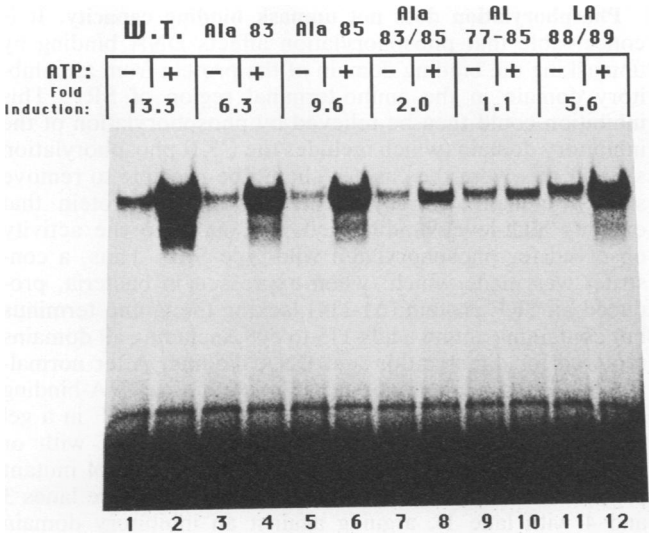

FIG. 2. Effect of mutations on enhancement of SRF DNA-binding activity. Bacterially made wild-type (W.T.) or mutant SRF proteins (as indicated; approximately 0.5 ng) were incubated with a phosphocellulose/MonoQ column-purified preparation of CKII (2 μl) in the presence (+) or absence (−) of 0.4 mM ATP and were assayed for DNA-binding activity, using the gel mobility shift assay. Quantitation of the fold induction of SRF DNA binding is given above each pair of lanes.

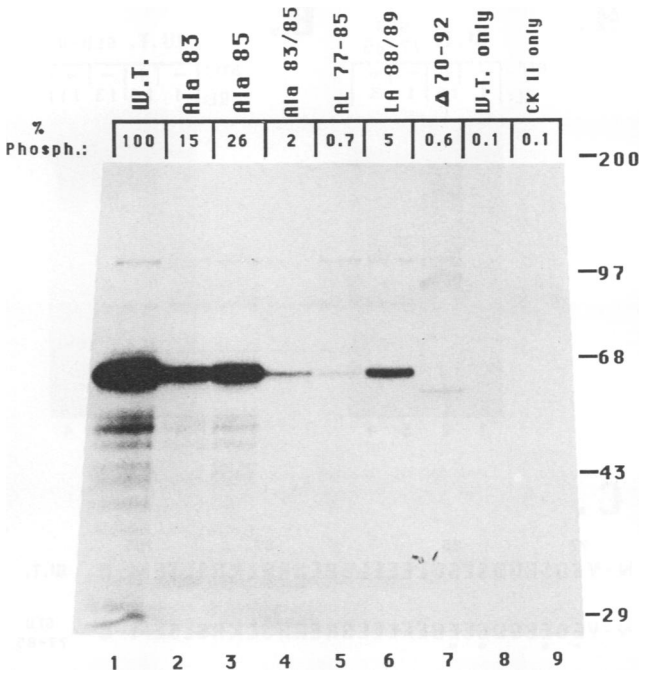

FIG. 3. Effect of mutations on SRF phosphorylation. Approximately 12 ng of the indicated BSRF proteins in a crude bacterial lysate was incubated with CKII (as in Fig. 2) in the presence of [γ-^{32}P]ATP and electrophoresed on an 8% SDS–polyacrylamide gel. The relative levels of phosphorylation are indicated above each lane. The sizes of marker proteins, in kilodaltons, are indicated to the right of the gel. W.T., wild type.

by a string of acidic amino acids (12), we reasoned that changing glutamate 88 and 89 to leucine and alanine, respectively, might have the desired effect. While the enhancement of DNA binding and phosphorylation was lowered, significant levels were still observed (Fig. 2, lanes 11 and 12 and Fig. 3, lane 6). Although phosphorylation was reduced to 5% of wild-type levels, it is clear that the CKII recognition sequence is more complex than expected. This low level of phosphorylation gave a significant (5.6-fold) increase in binding, again demonstrating that low levels of phosphorylation can give large increases in binding activity.

A mutation in the region of the CKII site mimics the phosphorylation effect. The complete loss of inducible binding activity with the AL 77-85 mutant allowed us to try to change this mutant protein to one which has a constitutively high level of DNA-binding activity. Since phosphorylation of serines creates a negatively charged region, we reasoned that we might be able to mimic the enhanced binding effect by creating a mutant that contained negatively charged glutamate residues in place of the serines normally in this region. A mutant in which all four serines were changed to glutamates was constructed (Glu-77-85; Fig. 4C), and the corresponding mutant protein was produced in bacteria. The DNA-binding activity of the mutant protein was compared with an equivalent amount of wild-type bacterially made SRF after incubation with CKII in the presence or absence of ATP (Fig. 4A). By comparing lanes 3 and 4 with lane 2, it can be seen that the Glu-77-85 protein did not exhibit a constitutively high level of binding. Moreover, the mutant exhibited only a threefold enhancement of binding in the presence of ATP (compare lanes 3 and 4). We found that this mutant was phosphorylated at low levels by CKII (4% of wild-type SRF levels; data not shown), such that the slight enhancement of DNA-binding activity may be due to the usage of a new, albeit poor, CKII phosphorylation site (the threonines at positions 66, 67, and 71 are potential phosphorylation sites). While the Glu-77-85 mutant did not exhibit constitutively high-level binding, we isolated a mutant during our mutagenesis screen that did. This mutant, Glu-X, had fortuitously been mutated in a number of additional positions that resulted in additional acidic residues being inserted, along with a 6-amino-acid deletion (Fig. 4C). When equivalent amounts of protein were assayed, the Glu-X mutant protein exhibited high-level binding, comparable with that of phosphorylated wild-type SRF, in the presence or absence of ATP (Fig. 4B). Thus, the Glu-X mutations were able to mimic the effect of phosphorylation on the wild-type protein, while simply changing serines 77 to 85 to negatively charged amino acids was not sufficient.

Phosphorylation does not affect dimerization. Since dimerization of SRF is required for its DNA-binding activity (16), one plausible explanation to account for phosphorylation enhancing SRF's DNA-binding activity is that phosphorylation of SRF monomers promotes the formation of dimers. Shuman et al. (24) have demonstrated that dimers of the transcription factor C/EBP can dissociate (and reassociate) within 1 min; therefore, we thought it possible that SRF subunit association might be stabilized by phosphorylation. We generated a carboxyl-terminal deletion mutant, Δ339-508, which retained its ability to bind DNA but exhibited a lower band in the gel mobility shift assay (Fig. 5A, lane 2 versus lane 1). Formation of a heterodimer of wild-type SRF with Δ339-508 would be expected to form a protein-DNA complex of intermediate mobility. This is exactly what we found when we denatured and renatured wild-type and Δ339-508 SRFs together (lane 4; the lower intensity of the

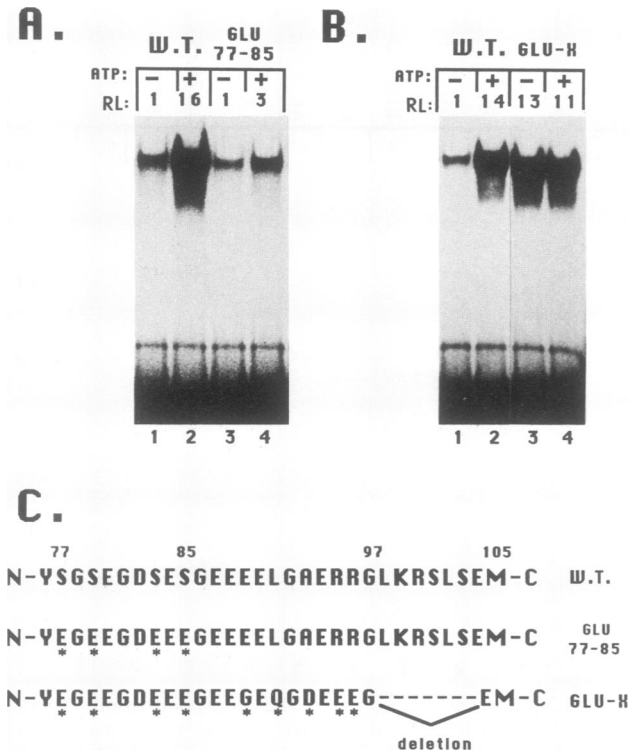

FIG. 4. DNA-binding activity levels of the Glu-77-85 and Glu-X mutants. Bacterially made SRF (W.T.) and either the Glu-77-85 (A) or Glu-X (B) mutant SRF proteins (as indicated; approximately 0.5 ng) were incubated with the partially purified CKII preparation (as in Fig. 2) in the presence (+) or absence (−) of 0.4 mM ATP and were assayed for DNA-binding activity, using the gel mobility shift assay. The relative levels (RL) of probe bound by the SRF proteins compared with the uninduced binding level of wild-type protein (lane 1) are indicated above the lanes. (C) Amino acid sequences of wild-type (W.T.), Glu-77-85, and Glu-X proteins within the region of the mutations. Amino acid positions are indicated by the numbers above the sequences. The amino acid changes in the mutants relative to the wild type are indicated by asterisks. The 6-amino-acid deletion in the Glu-X mutant is indicated.

Δ339-508 homodimer band is because less of this protein was used relative to wild-type SRF during the renaturation compared with the other lanes). Therefore, we set up an assay whereby we incubated together wild-type and Δ339-508 SRFs (separately prepared) for 30 min, either phosphorylating both proteins or neither and then performing a gel mobility shift assay. If there were an exchange of subunits of SRF dimers, we would expect to observe protein-DNA complexes of intermediate mobility containing wild-type Δ339-508 heterodimers. However, we were not able to detect the formation of any such heterodimers (lane 3), which suggests that SRF dimer formation is stable and that little dissociation to the monomer form occurs. Since unphosphorylated SRF appears to be entirely and stably in the dimer form, phosphorylation cannot increase SRF's dimerization significantly. Consistent with this, no effect on dimerization was observed if the proteins were phosphorylated (Fig. 5A, lanes 4 to 8; note that more SRF was used in lanes 1 to 4 than in lanes 5 to 8 in order to give similar signals). These results demonstrate that phosphorylation does not increase SRF's DNA-binding activity by increasing the stability of SRF dimers.

Phosphorylation does not unmask binding capacity. It is conceivable that phosphorylation affects DNA binding by unmasking the binding domain of the protein from an inhibitory domain in the amino-terminal region of SRF. This inhibition could then be relieved by phosphorylation of the inhibitory domain (which includes the CKII phosphorylation site). If this were the case, it should be possible to remove such a domain, thereby creating a mutant protein that exhibits high-level binding activity similar to the activity observed for phosphorylated wild-type SRF. Thus, a construct was made which, when expressed in bacteria, produced an SRF protein (Δ1-114) lacking the amino terminus but containing amino acids 115 to 508, including all domains required for dimerization and DNA binding. After normalization for protein amounts, this mutant's DNA-binding activity was compared with that of wild-type SRF in a gel mobility shift assay after incubation with CKII with or without ATP. As is evident in Fig. 5B, the Δ1-114 mutant protein exhibited only basal-level binding (compare lanes 3 and 4 with lane 1), arguing against an inhibitory domain model. (While in this experiment the basal binding of Δ1-114 was slightly decreased, it was not so in other trials.) It is still possible that another domain of the protein inhibits binding and that this is relieved by phosphorylation of the amino-terminal domain. We can only rule out the C-terminal domain as being inhibitory, since the Δ339-508 mutant still exhibited inducible binding (Fig. 5A and data not shown).

Change in conformation upon phosphorylation. Another mechanism by which phosphorylation might increase DNA-binding activity is via a conformational change in SRF upon phosphorylation. To test this idea, we performed partial tryptic proteolysis experiments on SRF which had been phosphorylated with CKII or left unphosphorylated. The partially digested products were electrophoresed on an SDS-polyacrylamide gel and subsequently visualized by immunoblotting. Antisera generated against a carboxyl-terminal synthetic peptide was used such that only digestion products retaining the C terminus of SRF were visualized. Interestingly, the unphosphorylated and phosphorylated protein digestions showed different cleavage patterns. With phosphorylated SRF, a band of altered mobility was observed at about 38 kDa and a new band was observed at about 34 kDa (note arrows in Fig. 6 and compare lanes 1 to 6 with 7 to 12). Since the antiserum used in the immunoblot analysis recognizes the C terminus of SRF, these sites of cleavage can be mapped to 38 and 34 kDa from the C terminus, respectively. Full-length SRF migrates to 64 kDa such that the sites of cleavage correspond roughly to the DNA-binding domain (Fig. 1). Note that the altered mobilities of the two fragments cannot be due to actual phosphorylation of the 34- and 38-kDa peptides, since these peptides do not span the phosphorylation site. Thus, phosphorylation of BSRF (at or around amino acid 83) appears to change the conformation of the protein at a distant site, the very region necessary for binding DNA (amino acids 133 to 222).

DISCUSSION

SRF is one of the few transcription factors whose DNA-binding activity has been shown to be increased by phosphorylation (13, 15, 19, 22, 30). We have now mutated the sites of SRF phosphorylation and found that these mutations also affect the enhancement of SRF's DNA-binding activity. We have further shown that phosphorylation of SRF affects the conformation of the protein.

Site of SRF phosphorylation. We have previously shown

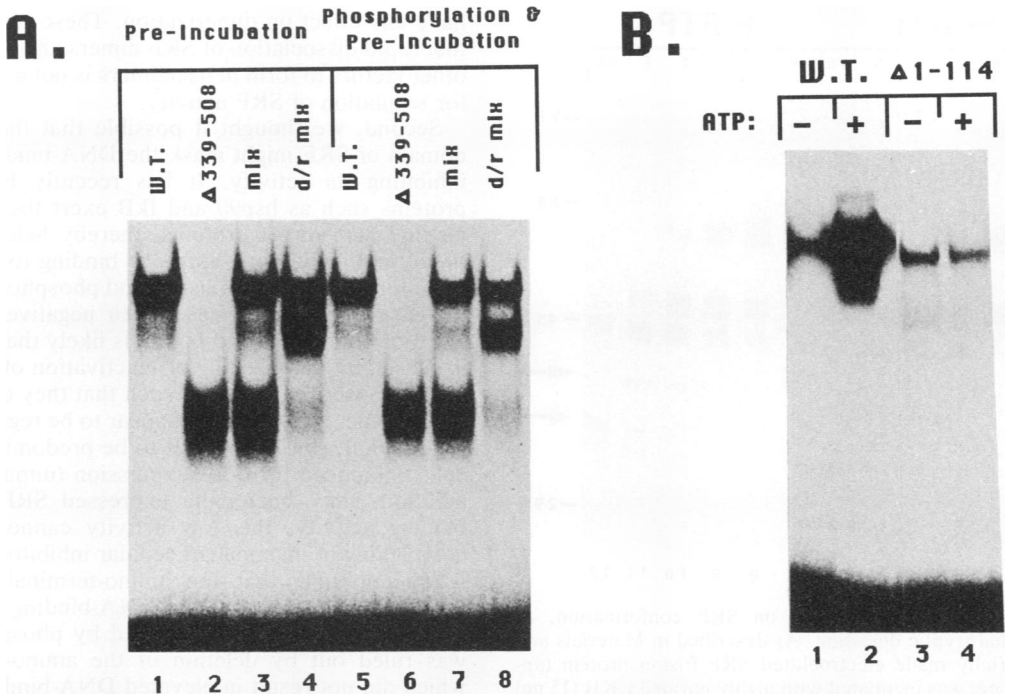

FIG. 5. Dimerization and masking domains. (A) Effect of phosphorylation on dimerization. Bacterially made full-length SRF (W.T.) and a carboxyl-terminal deletion mutant (Δ339-508) were assayed separately (lanes 1, 2, 5, and 6) or together, either by mixing the proteins (mix, lanes 3 and 7) or denaturing and renaturing them together (d/r mix, lanes 4 and 8). These proteins were preincubated with (lanes 5 to 8) or without (lanes 1 to 4) CKII (see Fig. 2) and 0.4 mM ATP for 30 min at 37°C, followed by the gel mobility shift assay. Approximately 0.5 ng (lanes 5 to 8) and 10 ng (lanes 1 to 4) of the bacterially made proteins were used per reaction in order to give similar signals. (B) Effect of deletion of the amino-terminal domain on the activity of the DNA-binding domain. Bacterially made SRF (W.T.; 0.5 ng) and an amino-terminal deletion mutant (Δ1-114; 0.5 ng) were incubated with CKII in the presence (+) or absence (−) of ATP (as in Fig. 2) and assayed for DNA-binding activity, using a gel mobility shift assay.

that SRF is phosphorylated by CKII and that this phosphorylation results in a dramatic increase in SRF's DNA-binding activity (13). In this paper, we have established that phosphorylation by CKII on serine residues within the region of amino acids 77 to 85 is solely responsible for the enhancement of SRF's DNA-binding activity. Serine 85 is located N terminal to a stretch of four glutamates, indicative of a CKII consensus recognition sequence (12); however, it appears that serine 83 is more important, since mutation of this amino acid had a greater effect on phosphorylation and enhanced DNA binding than mutation of serine 85. In addition, our preliminary result from sequencing the phosphorylated SRF tryptic peptide is that serine 83 is the predominant amino acid that is phosphorylated (1a).

Although mutation of serine 83 has the greatest effect, it does not abolish phosphorylation. While it appears to be the preferred site of phosphorylation, we believe that when serine 83 is mutated, serine 85 can substitute for it and be efficiently phosphorylated. Mutation of serine 85 alone had a smaller but significant effect on phosphorylation that may be due to alteration in the protein kinase recognition site. Double mutation of serines 83 and 85 caused a much greater reduction in phosphorylation than mutation of either alone. Nevertheless, additional mutation of serines 77 and 79 was required before phosphorylation and enhanced binding were completely abolished. Thus, serines 77 and 79 could also be recognized by CKII if serines 83 and 85 were mutated. CKII could also phosphorylate, albeit poorly, a mutant (LA 88/89) which had two of the glutamates in its recognition sequence altered. These results suggest that the recognition sequence for CKII phosphorylation is more complex than expected. This is not to say that CKII is not highly specific, since SRF was efficiently phosphorylated even when it was present as a minor component of a crude bacterial lysate.

While our preliminary sequencing results suggest that only serine 83 is phosphorylated on SRF in vitro, we cannot say exactly which one(s) of these serines is phosphorylated in vivo. We have previously found, however, that the same tryptic peptide of SRF is phosphorylated in vivo as in vitro (13). We are currently investigating, using two-dimensional phosphotryptic peptide mapping, whether more than one site is phosphorylated at the same time in vivo and whether phosphorylation at these specific sites varies with induction of the c-*fos* gene by growth factors.

Once we had isolated the AL 77-85 mutant whose protein product had absolutely no enhanced binding in response to CKII, we were interested in further generating a mutant that would have constitutively high DNA-binding activity. Since phosphorylation of SRF introduces a negative charge, we sought to mimic this effect by changing all four serines at positions 77, 79, 83, and 85 to glutamates. However, such a mutant did not exhibit a constitutively high level of DNA-binding activity comparable with that of phosphorylated wild-type SRF. Nonetheless, we were fortuitously able to isolate a mutant, Glu-X, which did possess this property. It exhibited high-level DNA binding without phosphorylation which could not be raised further by CKII in the presence of ATP. When sequenced, the Glu-X mutant was found to contain, in addition to glutamate residues in place of serines 77 to 85, a number of downstream missense mutations as

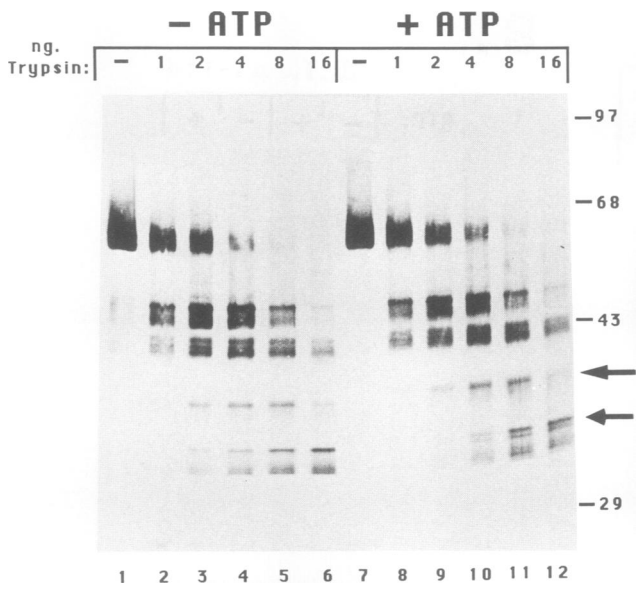

FIG. 6. Effect of phosphorylation on SRF conformation, as revealed by partial tryptic digestion. As described in Materials and Methods, bacterially made electroeluted SRF fusion protein (approximately 100 ng) was incubated with highly purified CKII (15 ng) in the presence (lanes 7 to 12) or absence (lanes 1 to 6) of ATP. The samples were digested with the indicated amounts of trypsin, electrophoresed on a 10% SDS–polyacrylamide gel, and immunoblotted using an affinity-purified antiserum directed against the C terminus of SRF. The sizes of marker proteins, in kilodaltons, are indicated to the right of the gel.

well as a 6-amino-acid deletion. Clearly, changing the serines to negatively charged glutamates was insufficient to mimic the effect of phosphorylation, although in conjunction with the additional Glu-X mutations, enhanced binding was observed. The location of the Glu-X mutations in the same region as the CKII phosphorylation site further demonstrates that changes in this domain of SRF affect the activity of the DNA-binding domain. We are now in the position to use this mutant to check whether CKII phosphorylation of SRF is a step in the regulation of c-*fos* transcription in vivo. If so, we would predict that introduction of the Glu-X SRF gene into cells will cause increased c-*fos* gene expression in the absence of normal activators such as serum. The Glu-X mutations affect DNA-binding activity but should not affect SRF's ability to activate transcription, since we have found that the Glu-X protein activates transcription in vitro just as well as wild-type SRF does (18a).

Mechanism of activation of SRF's DNA-binding activity. While phosphorylation of SRF occurs at or around amino acid 83, it affects the activity of the DNA-binding domain over 50 amino acids away (amino acids 133 to 264). We investigated several possibilities which might explain this effect. First, phosphorylation might affect SRF dimerization which is required for SRF's DNA-binding activity (16). The C/EBP protein, a leucine zipper-containing transcription factor that regulates gene expression in a variety of tissues, binds to its recognition sites as a dimer; furthermore, C/EBP subunits have been shown to exchange readily in the absence of DNA (24). We do not observe any such effect for SRF in solution, thereby suggesting that SRF, once synthesized, dimerizes and remains stable as a dimer in solution. As expected, phosphorylation of the SRF dimers did not have any effect on dimerization. These results also suggest that rapid dissociation of SRF dimers and reassociation with other factors to form heterodimers is not a likely mechanism for regulation of SRF activity.

Second, we thought it possible that the amino-terminal domain of SRF might mask the DNA-binding domain, thus inhibiting its activity. It has recently been shown that proteins such as hsp90 and IkB exert their effect by complexing with target proteins, thereby helping to inactivate them until a stimulus (hormone binding to steroid hormone receptors in the case of hsp90 and phosphorylation of IkB in the case of NF-κB) releases their negative influence (4, 17, 18). For both hsp90 and IkB, it is likely that their repression is caused by the masking or inactivation of critical domains of the transcription factors such that they cannot localize to the nucleus. SRF does not appear to be regulated by cellular localization, since we find it to be predominantly nuclear in cells uninduced for c-*fos* expression (unpublished data). In addition, since bacterially expressed SRF has low DNA-binding activity, this low activity cannot be due to the complexing of mammalian cellular inhibitors. Nevertheless, it was possible that the amino-terminal region of SRF inhibited the activity of the DNA-binding domain and that this inhibition would be relieved by phosphorylation. This was ruled out by deletion of the amino-terminal domain which did not result in elevated DNA-binding activity.

A third possibility was that phosphorylation altered the conformation of SRF, resulting in increased DNA-binding activity. SRF's structure was probed by partial tryptic digestion. Changes in the preferred sites of cleavage were observed after phosphorylation of SRF. These altered sites localized to the general region of the DNA-binding domain. Moreover, we have also observed changes in cleavage patterns when other endoproteases (chymotrypsin and V8) have been used (data not shown). Therefore, phosphorylation at (or around) amino acid 83 appears to cause a conformational change in the DNA-binding domain over 50 amino acids away. Clearly, a finer structural analysis will be required to determine the cause and nature of the conformational change.

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