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Identification of multiple SRF N-terminal phosphorylation sites affecting DNA binding properties

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Human serum response factor (SRF) bearing a histidine tag was expressed using vaccinia virus. The recombinant protein was purified and shown to be phosphorylated mainly in its N-terminal part. The corresponding phosphorylation sites were mapped by microsequencing and also appear to be phosphorylated in endogenous serum response factor. Four phosphorylation sites are located on serines within amino acids 77-85, while another phosphorylation site has been identified at Ser103. Mutations that considerably reduced or abolished phosphorylation at amino acids 77-85 caused a decrease in binding to the c-*fos* serum response element accompanied by markedly reduced association and dissociation rates. In contrast, replacing Ser103 by alanine decreased DNA binding activity without drastically affecting the on/off rates. The combination of abolishing phosphorylation at amino acids 77-85 and 103 displayed greatly reduced on/off rates of DNA binding, but the reduction of DNA binding activity was partially alleviated. None of these mutations affect either the ability to interact with p62^{TCF} or stimulation of transcription *in vitro*. These findings imply possible roles for SRF phosphorylation in the regulation of c-*fos* transcription.
Key words: c-*fos*/Ni^{2+}-NTA/phosphorylation/serum response factor/vaccinia virus

Introduction

The c-*fos* proto-oncogene is a member of the family of immediate-early genes, which are rapidly induced after treatment of quiescent cells with growth factors or mitogens (Almendral *et al.*, 1988). Many of the immediate-early genes, including c-*fos*, encode transcription factors that are thought to relay signals generated by extracellular stimuli to target genes in a cell-type specific fashion (Cohen and Curran, 1989). Deciphering the molecular events leading to the activation of the c-*fos* gene will further our mechanistic understanding of the induction of immediate-early genes and thereby elucidate an initial nuclear step in the response of cells to extracellular stimuli.

The serum response element (SRE), which is located 300 bp upstream of the c-*fos* transcription initiation site, is of pivotal importance for the induction of c-*fos* transcription by several agents, e.g. serum, epidermal growth factor or phorbol esters (Treisman, 1985; Fisch *et al.*, 1987; for review: Rivera and Greenberg, 1990). In addition, the SRE mediates the subsequent repression of the c-*fos* promoter occurring ~30 min after induction, as well as c-*fos* inactivity prior to induction (König *et al.*, 1989; Shaw *et al.*, 1989a). Several proteins have been described to interact with the SRE, of which the roles of the 67 kDa serum response factor (SRF) and p62^{TCF} are the best characterized (Treisman, 1986; Norman *et al.*, 1988; Ryan *et al.*, 1989; Shaw *et al.*, 1989b). The SRF binds as a dimer independent of p62^{TCF} to the SRE, while the p62^{TCF} protein is unable to interact with the SRE in the absence of SRF (Schröter *et al.*, 1990). Both proteins seem to be indispensable for full function of the SRE (Shaw *et al.*, 1989b; Graham and Gilman, 1991).

Genomic footprinting experiments provide evidence that the SRE is constitutively occupied by SRF before, during and after induction (Herrera *et al.*, 1989). Activation of c-*fos* transcription via the SRE is therefore probably caused by the recruitment of SRF associated factors or by post-translational modification of SRF or its associated proteins. Since SRF was shown to be post-translationally modified by phosphorylation, this might correlate with SRE/SRF mediated induction of c-*fos*. Phosphorylation is a necessary prerequisite for the DNA binding activity of SRF and is apparently constitutive (Prywes *et al.*, 1988). The degree of SRF phosphorylation seems to be variable (Misra *et al.*, 1991) and was shown to increase in transformed mouse embryo fibroblasts upon induction with epidermal growth factor (Schalasta and Doppler, 1990). Thus, it appears likely that SRF activity could be regulated by phosphorylation, as suggested for other transcription factors such as CREB (Gonzalez and Montminy, 1989), Oct-2 (Tanaka and Herr, 1990) or c-Jun (Binétruy *et al.*, 1991).

In order to determine the sites of SRF phosphorylation and their influence on SRF activity, we have purified SRF tagged with a stretch of six histidines (SRF-6His) from HeLa cells infected with recombinant vaccinia virus. Peptides of SRF-6His bearing major phosphorylation sites were isolated. Subsequent microsequencing revealed five major phosphorylation sites in SRF-6His, all of them located in the N-terminal region. To study the impact of phosphorylation at the different sites on the activity of SRF, mutant forms of SRF-6His lacking phosphorylation sites were generated and their DNA binding and *in vitro* transcriptional activities were compared with SRF-6His. The results indicate that phosphorylation in two different regions substantially influences SRE-SRF interaction and might accordingly contribute to c-*fos* transcriptional regulation.

Results

Vaccinia expressed SRF-6His is mainly phosphorylated in its N-terminal domain

To obtain sufficient amounts of SRF for phosphoamino acid mapping, we expressed SRF-6His with recombinant vaccinia virus. The SRF-6His protein contains an N-terminal tag of

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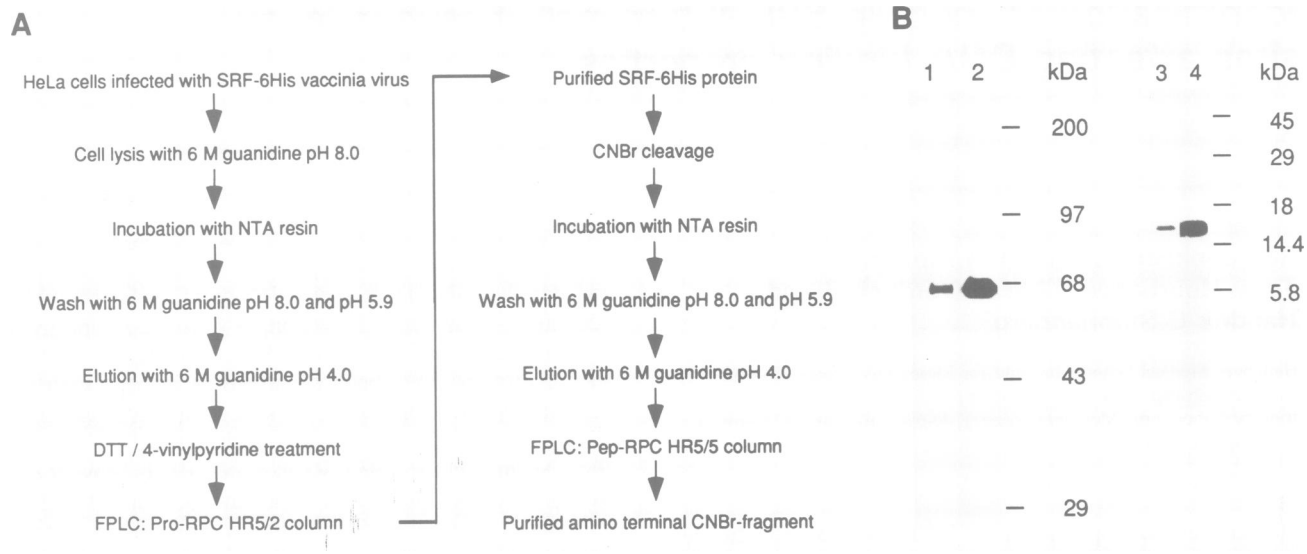


Fig. 1. Purification of SRF-6His and its N-terminal CNBr fragment. (**A**) Schematic outline of the purification procedure. (**B**) Silver-stained protein gel showing purified SRF-6His (lane 1) or its N-terminal CNBr fragment (lane 3). Lanes 2 and 4 show the corresponding autoradiograms. The apparent molecular weight of SRF-6His and its N-terminal CNBr fragment are 67 and 16 kDa, respectively.

six histidines linked to the SRF protein missing its first nine amino acids. The histidine tag allows a rapid purification of SRF-6His by means of Ni^{2+}-NTA affinity chromatography under denaturing conditions (Hochuli *et al.*, 1988). Previously, we have shown SRF-6His to be indistinguishable from SRF by a variety of structural and functional criteria (Janknecht *et al.*, 1991). Thus, HeLa monolayer cells infected with recombinant SRF-6His vaccinia virus were labelled with $^{32}P_i$ and lysed with 6 M guanidine pH 8.0 buffer, which leads to an instantaneous inactivation of phosphatases and proteases. SRF-6His protein was purified by Ni^{2+}-NTA affinity and reverse phase chromatography, as outlined in Figure 1A. The yield of purified SRF-6His was $\sim 5-10$ μg per 9 cm dish of HeLa cells. As judged from a silver-stained protein gel and the corresponding autoradiogram, SRF-6His was >95% pure and no major phosphorylated contaminant could be observed (Figure 1B).

This purified SRF-6His was subsequently cleaved with CNBr and the resulting peptides were separated by Ni^{2+}-NTA affinity chromatography (Figure 1A). Eighty-five percent of the radioactivity was specifically bound to the NTA resin indicating that the histidine-tagged N-terminal CNBr fragment contains the major phosphorylation sites of SRF-6His. This peptide was eluted from the Ni^{2+}-NTA column with 6 M guanidine buffer at pH 4.0 and further purified by reverse phase chromatography. Figure 1B shows the purity of this peptide. No major contaminating peptide could be detected on a protein gel or on the corresponding autoradiogram. Phosphoamino acid analysis of the purified N-terminal CNBr fragment revealed that phosphorylation was confined to serine residues (Figure 2) in agreement with the phosphoamino acid analysis of native SRF (Prywes *et al.*, 1988).

Comparison of phosphorylation sites in transfected and in vaccinia virus infected HeLa cells

To rule out that vaccinia virus kinases or phosphatases are responsible for the phosphorylation pattern observed in SRF-6His, we expressed a variant SRF-6His (tk-SRF-6His) by transiently transfecting HeLa cells. This was then

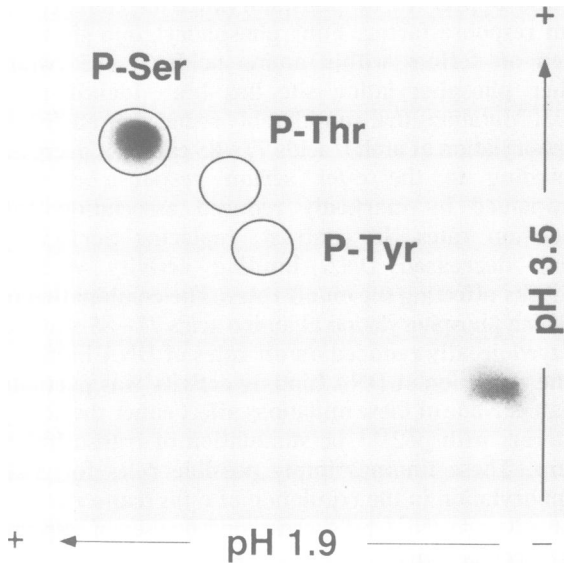


Fig. 2. Phosphoamino acid analysis of the N-terminal CNBr fragment. The N-terminal CNBr fragment of SRF-6His was cleaved with 6 M HCl. Resulting phosphoamino acids were separated by two-dimensional thin-layer electrophoresis (1st dimension: pH 1.9; 2nd dimension: pH 3.5) and revealed by autoradiography. Circles denote the position of co-electrophoresed phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr), which were visualized by ninhydrin staining.

compared with SRF-6His derived from HeLa cells infected with recombinant vaccinia virus. The tk-SRF-6His differs from the vaccina expressed SRF-6His only by 13 additional amino acids at the N-terminus.

Transfected cells were either continuously grown in the presence of serum or serum-starved and then induced by serum addition. These cells were labelled with $^{32}P_i$ and the tk-SRF-6His proteins and their N-terminal CNBr fragments were purified as described for the vaccinia expressed SRF-6His. The amounts of tk-SRF-6His protein isolated from equal numbers of HeLa cells were $\sim$5- to 10-fold lower than SRF-6His. The purified N-terminal CNBr

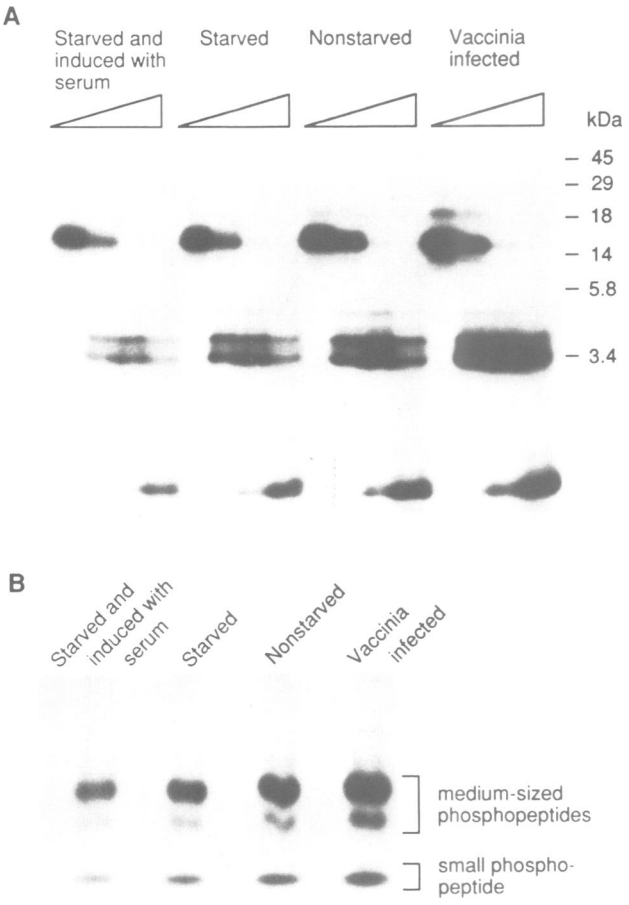


Fig. 3. Phosphorylation pattern of the N-terminal CNBr fragment in transfected HeLa cells. (**A**) HeLa cells were transfected with tk-SRF-6His and then continuously grown in the presence of serum (Non-starved) or deprived of serum (Starved) and then induced for 25 min with serum (Starved and induced with serum). ^{32}P-labelled N-terminal CNBr fragments of tk-SRF-6His were purified and compared with the respective fragment of SRF-6His purified from recombinant vaccinia virus infected cells (Vaccinia infected). The N-terminal CNBr fragments were digested with increasing amounts of Glu-C as indicated. The resulting phosphopeptides were separated by SDS−PAGE and visualized by autoradiography. (**B**) Corresponding trypsin cleavage of the N-terminal CNBr fragments.

fragments of the tk-SRF-6His proteins, as well as of SRF-6His, were digested with increasing amounts of Glu-C (Figure 3A). The pattern of the cleavage products was the same for all of the N-terminal CNBr fragments. This similarity was confirmed by cleaving the N-terminal CNBr fragments with trypsin (Figure 3B). As will be demonstrated later, the medium-sized tryptic phosphopeptides reflect phosphorylation at several serines co-localized in a short stretch, while the small tryptic phosphopeptide reflects phosphorylation at a different serine. The ratio of phosphorylation between the small and medium-sized phosphopeptides did not vary between the tk-SRF-6His and the vaccinia SRF-6His. These findings imply that the phosphorylation pattern observed for SRF-6His is not substantially influenced by the vaccinia virus expression system. Furthermore, the N-terminal phosphorylation of tk-SRF-6His is apparently unaltered upon serum induction of transfected HeLa cells, which has also been observed for SRF-6His in vaccinia virus infected HeLa cells (data not shown).

Mapping of phosphorylation sites

Preliminary investigations localized the major phosphorylation sites to the second half of the SRF-6His N-terminal CNBr fragment (data not shown). Thus, serines −16, 16 and 21 (see Figure 4A) are not phosphorylated to detectable levels, which was confirmed by the analysis of mutant derivatives of SRF-6His (see below). This left only the serines at positions 77, 79, 83, 85, 101 or 103 as possible major phosphorylation sites.

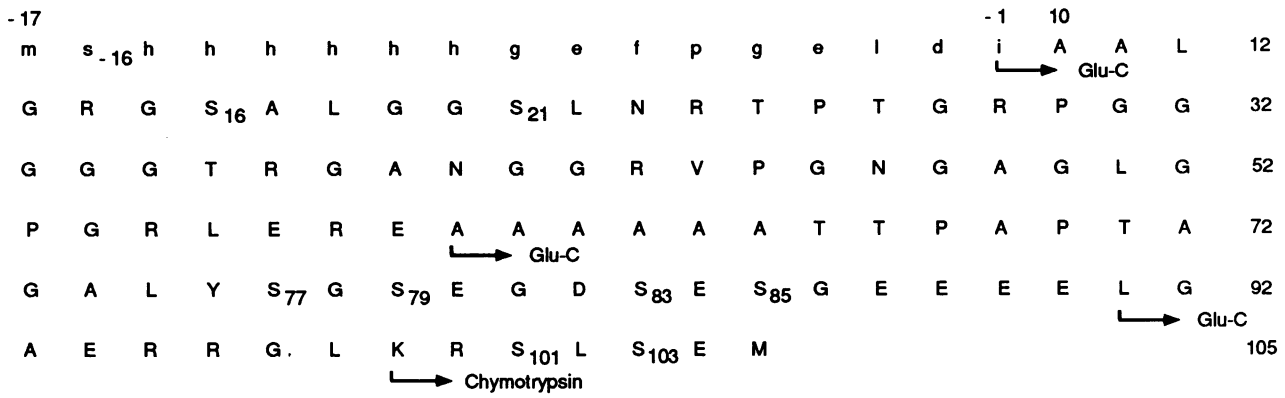
To generate smaller phosphopeptides, we first cleaved the N-terminal CNBr fragment of SRF-6His with chymotrypsin. One small phosphopeptide could be isolated using reverse phase chromatography sufficiently pure to allow microsequencing. This identified the peptide to span amino acids 99−105 (Figure 4A and B). The peptide contains two serines (positions 101 and 103) as potential phosphorylation sites. However, the reduced yield of Ser103 observed at cycle 5 of the microsequencing procedure (27 pmol), in comparison with all other cycles (68−227 pmol) and especially in comparison with Ser101 at cycle 3 (87 pmol), suggested that only Ser103 was phosphorylated in that peptide.

To clarify this point and to map additional phosphorylation sites, we performed a Glu-C cleavage of the N-terminal CNBr fragment and obtained a mixture of phosphopeptides after reverse phase chromatography. Prior to sequencing these phosphopeptides were modified with ethanethiol, which converts phosphoserine residues into S-ethylcysteine residues (Meyer *et al.*, 1986). This permits the direct detection of phosphoserine via S-ethylcysteine with standard sequencing procedures. Microsequencing of the mixture of Glu-C peptides revealed three peptides that started at amino acid −1, 60 or 91 (Figure 4A and C). No S-ethylcysteine was observed at cycle 11 of the microsequencing procedure, once again indicating that Ser101 is not detectably phosphorylated. In contrast, S-ethylcysteine was found at cycle 13, which could coincide with the modification of Ser21, Ser103 or both of them (see peptides 2 and 3, Figure 4C). Since our preliminary investigations excluded phosphorylation of Ser21 (data not shown), this confirmed Ser103 to be phosphorylated. Additional S-ethylcysteines were observed at cycles 18, 20 and 24, which corresponded to Ser77, 79 and 83, respectively. Due to a low signal to noise ratio we could not unambiguously determine whether S-ethylcysteine was present at cycle 26, which would correspond to Ser85. Therefore, a peptide starting at amino acid 83 was sequenced. This peptide was generated by partial formic acid cleavage of the N-terminal CNBr fragment after aspartic acid residues, purified by reverse phase chromatography and modified with ethanethiol. Subsequent microsequencing showed S-ethylcysteine at cycle 3 (data not shown) indicating that Ser85 was phosphorylated. Thus, the N-terminal CNBr fragment of SRF-6His contains five major phosphorylation sites: Ser77, 79, 83, 85 and 103.

Phosphorylation of mutant derivatives of SRF-6His

We introduced a series of mutations into SRF-6His (Figure 5) with the aim of affecting its phosphorylation status. Recombinant vaccinia viruses were generated expressing these mutant forms of SRF-6His. Using the strategy described above, SRF-6His mutants M1−M6 were isolated from HeLa cells labelled with $^{32}P_i$. Their purified

A



B

Cycle	Amino Acid (pmol)
1	K (227)
2	R (68)
3	S (87)
4	L (143)
5	S (27)
6	E (79)

C

Cycle	Amino Acids (pmol) Peptide 1	Peptide 2	Peptide 3
1	A (13)	i (202)	L (51)
2	← A	(291) →	G (38)
3	←	A (326)	→
4	A (156)	L (172)	E (26)
5	A (186)	G (147)	R (0.8)
6	A (163)	← R	(0) →
7	T (85)	← G	(101) →
8	T (50)	S (23)	L (18)
9	P (83)	A (61)	K (1.9)
10	A (73)	L (52)	R (0)
11	P (59)	G (48)	S (1.5)
12	T (29)	G (36)	L (2.4)
13	A (62)	← S*	(15) →

Cycle	Amino Acids (pmol) Peptide 1	Peptide 2	Peptide 3
14	G (47)	L (32)	E (3.9)
15	A (62)	N (22)	
16	L (27)	R (0)	
17	Y (11)	T (7.7)	
18	S* (0.5)	P (9.4)	
19	G (3.6)	T (3.3)	
20	S* (0)	G (0.9)	
21	E (2.1)	R (0.4)	
22	G (1.9)	P (6.9)	
23	D (0)	G (1.1)	
24	S* (0)	G (0)	
25	E (0.7)	G (0)	
26	S(*) (0.3)	G (3.0)	

Fig. 4. Microsequencing of SRF-6His phosphopeptides. (**A**) Amino acid sequence of the N-terminal CNBr fragment. Small letters indicate the artificial N-terminus that replaces the first nine amino acids of SRF. Positions of non-SRF amino acids are numbered from −17 to −1. The other numbering corresponds to authentic positions in SRF. Positions of all serines are numbered. Arrows indicate the start of microsequenced peptides derived from cleavages with chymotrypsin or Glu-C. (**B**) Amino acids determined at each cycle of the microsequencing reaction of a chymotryptic phosphopeptide. The amounts of amino acids are lag-corrected. (**C**) Microsequencing of a mixture of three peptides derived from cleavage of the N-terminal CNBr fragment by Glu-C. Asterisks denote the appearance of S-ethylcysteine in addition to serine. S-ethylcysteine was not quantitated.

N-terminal CNBr fragments were then cleaved with trypsin and the resulting phosphopeptides were separated by SDS−PAGE. Two classes of phosphopeptides were observed (Figure 6): a small phosphopeptide co-migrating with the salt front and a group of medium-sized phosphopeptides appearing as a broad band (sometimes resolved into two bands: compare with Figure 3B). Comparison of the predicted trypsin cleavage products with those obtained from the N-terminal CNBr fragments of SRF-6His-M4 and M6 identified the two classes of phosphopeptide. The small phosphopeptide corresponds to amino acids 101−105, because trypsin cleavage in the case of the Ala103 mutant SRF-6His-M6 produced only the medium-sized phosphopeptides. The medium-sized phosphopeptides must span amino acids 59−95, because trypsin cleavage in the case of SRF-6His-M4, which has alanines at positions 77, 79, 83 and 85, yielded only the small phosphopeptide. Since no other phosphopeptides were observed, phosphorylation in the N-terminal CNBr fragment must occur predominantly, if not exclusively, at position 77, 79, 83, 85 or 103.

Mutation of Ser77 and 79 to alanines (SRF-6His-M1) had little effect on the phosphorylation of the medium-sized

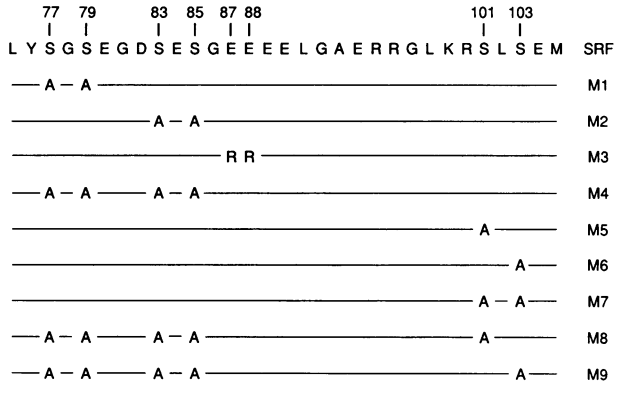


Fig. 5. Mutations introduced into SRF-6His. Amino acids 75−105 of SRF are depicted. The amino acid exchanges in SRF-6His mutants M1−M9 are indicated.

peptides, while it was dramatically diminished by mutation of Ser83 and 85 to alanines (SRF-6His-M2, Figure 6). The appearance of the figure is somewhat deceiving here, since the medium-sized phosphopeptides are faintly visible on the original autoradiogram. In fact, quantitation using a

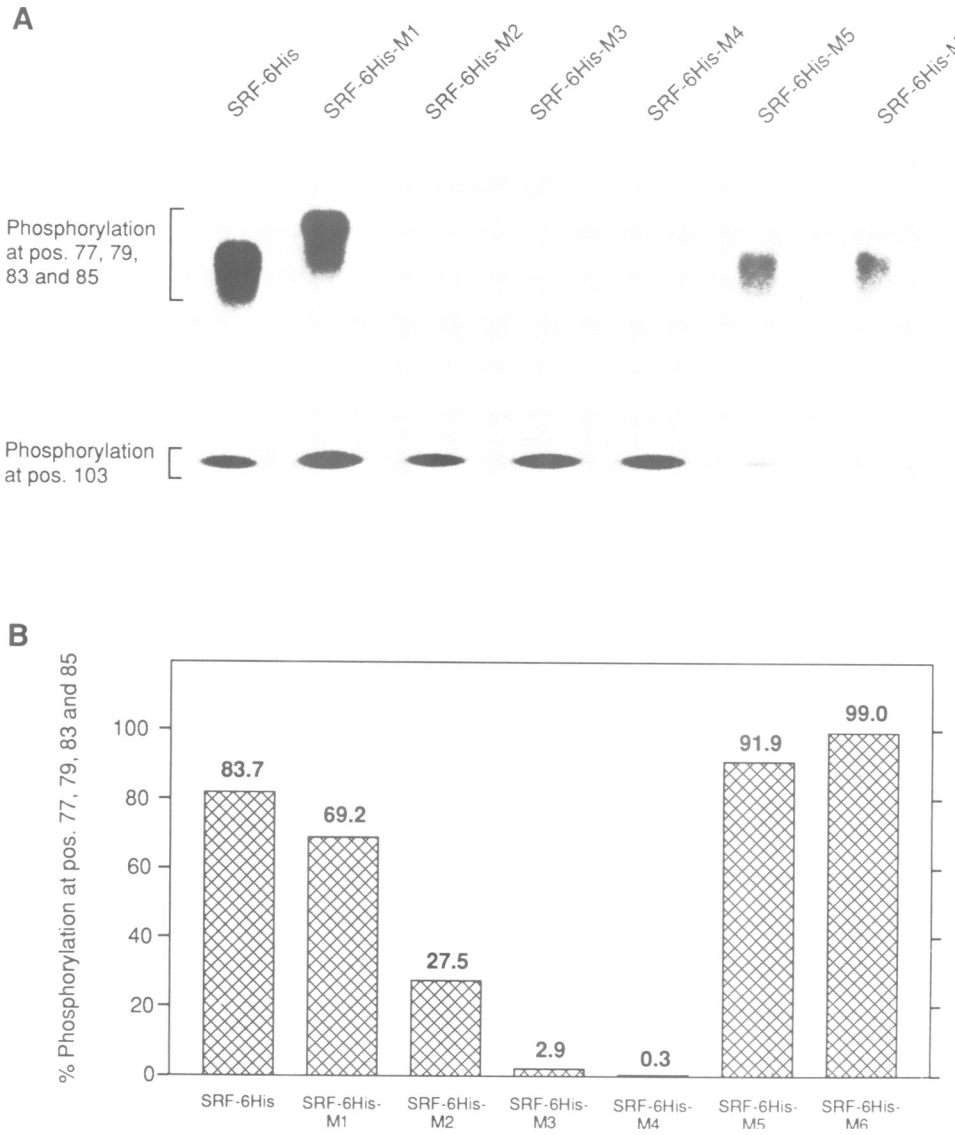


Fig. 6. Phosphorylation of SRF-6His mutants M1−M6. (**A**) The N-terminal CNBr fragments of the SRF-6His mutants were cleaved with trypsin. Resulting phosphopeptides were separated on a protein gel and visualized by autoradiography. The medium-sized phosphopeptides represent phosphoserines at positions 77, 79, 83 or 85. The small phosphopeptide represents phosphoserine at position 103. The part of the figure showing tryptic peptides of SRF-6His-M5 and M6 was derived from a separate gel, which was autoradiographed for a shorter time than the other gels shown. (**B**) ^{32}P incorporation in the small and medium-sized phosphopeptides was determined with the volume-integration function of the PhosphorImager (Molecular Dynamics) and corrected for background. Phosphorylation at positions 77, 79, 83 and 85 is given as a percentage of the total phosphorylation at positions 77−85 and position 103.

phosphoanalyser clearly showed a considerable amount of phosphorylation. These results suggest that Ser83/85 are more efficiently phosphorylated than Ser77/79.

Ser77, 79, 83 and 85 have been implicated in the phosphorylation of bacterially expressed SRF by casein kinase II (Manak *et al.*, 1990; Manak and Prywes, 1991). To test this, we replaced the two negatively charged residues at positions 87/88 with arginine (SRF-6His-M3), thereby destroying a potential casein kinase II recognition sequence. SRF-6His-M3 displayed only residual phosphorylation at Ser77, 79, 83 and 85. This demonstrates the importance of glutamic acid at positions 87/88 for those phosphorylation events and thus strongly suggests casein kinase II to be involved in the *in vivo* phosphorylation of Ser77, 79, 83 and 85.

Mutation of Ser101 to alanine (SRF-6His-M5) led to a 2-fold reduction of phosphorylation of the tryptic peptide 101−105 as compared with SRF-6His. Since Ser101 itself was not detectably phosphorylated, this reduction must reflect some influence of position 101 on the phosphorylation of Ser103. Interestingly, exchanging both Ser101 and 103 with alanine (SRF-6His-M7) generated an unstable protein. An N-terminal degradation product accumulated and had roughly the same size as the N-terminal CNBr fragment of SRF-6His (data not shown). When this N-terminal degradation product was cleaved with CNBr and trypsin, we could still observe a phosphopeptide pattern which indicated phosphorylation at positions 77, 79, 83 and 85 to be unaffected (data not shown).

Effects of phosphoserine mutations on DNA binding properties

To test our SRF-6His mutants for their DNA binding properties, we performed band shift assays investigating the association and dissociation kinetics of the binding to the

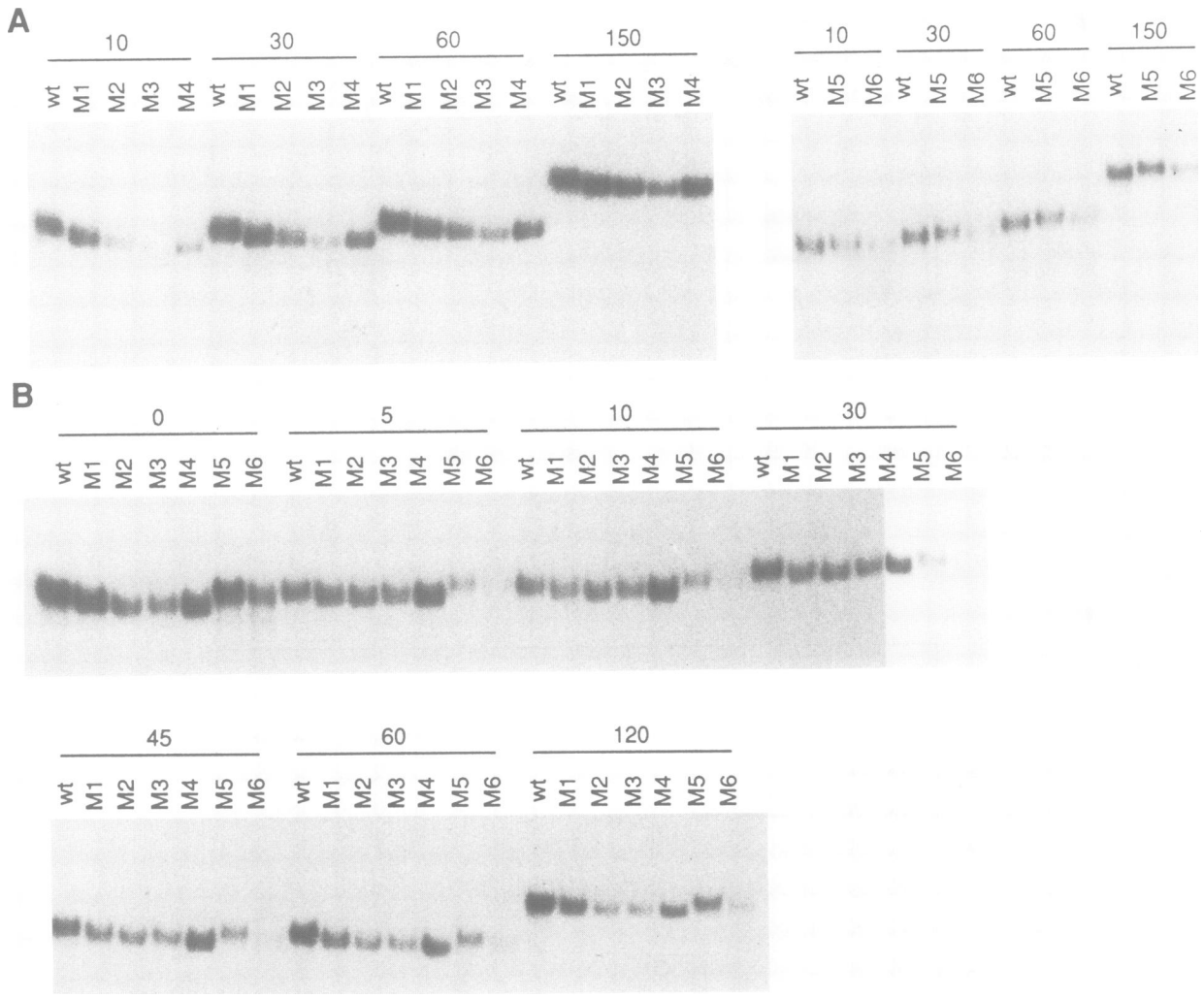


Fig. 7. DNA binding kinetics of SRF-6His mutants M1 to M6. (**A**) Binding of extracts containing mutated SRF-6His to a ^{32}P-labelled SRE was assayed by band shift gels. The time of binding prior to loading the gel is indicated (in minutes). Specific complexes of SRF-6His mutants with the SRE were revealed by autoradiography. Since the gel was continuously electrophoresed, the distance of complex migration is inversely related to the incubation time of the binding reaction. No more than 50% of the labelled SRE was complexed in any lane (not shown). wt, SRF-6His; M1–M6, SRF-6His mutants M1–M6. (**B**) Extracts containing SRF-6His mutants were incubated with a ^{32}P-labelled SRE overnight to attain equilibrium. Then an 8-fold excess of unlabelled SRE was added and aliquots removed for immediate gel electrophoresis at the indicated time points (in minutes). Once again, no more than 50% of the labelled SRE was complexed in any lane.

c-*fos* SRE. SRF-6His exhibited very rapid association with and dissociation from the SRE: it was bound to equilibrium levels within 10 min and it dissociated in the presence of an excess of unlabelled SRE within 5 min (Figures 7 and 8). Due to a lower excess of competitor, the decrease of SRE–SRF-6His complex formation within 5 min is less pronounced in Figure 7B than in Figure 8B. Mutation of Ser77 and 79 (SRF-6His-M1), which did not drastically reduce the phosphorylation state of amino acids 77–85, caused no visible change of DNA binding kinetics (Figure 7). Since the first measurements of association and dissociation were done at 10 and 5 min, respectively, and both SRF-6His and the mutant M1 exhibited very fast kinetics, we would not detect relatively subtle changes in the DNA binding kinetics. We next determined the alteration of DNA binding activity of the mutant M1 compared with SRF-6His. This was done by measuring the complex formation with the SRE at the 0 min time point of the dissociation kinetics and normalizing this by the amount of respective SRF-6His variant present in the binding reaction. When measured in that way, the DNA binding activity of SRF-6His-M1 was only slightly reduced to 88% of the level of SRF-6His (Table I).

Changing Ser83 and 85 to alanines (SRF-6His-M2), which greatly reduces the phosphorylation state of amino acids 77–85, resulted in markedly reduced on- and off-rates of DNA binding (Figure 7): SRF-6His-M2 took >30 min to associate with the SRE to equilibrium levels and >60 min to dissociate. Furthermore, the DNA binding activity was reduced to 38% of the level of SRF-6His (Table I). The behaviour of SRF-6His-M3 and M4 in DNA binding resembles that of SRF-6His-M2 (Figure 7 and Table I). Comparing the decrease of phosphorylation on amino acids 77–85 with the decrease of relative DNA binding activity for SRF-6His mutants M1–M4 reveals a correlation: the decrease in the phosphorylation state at positions 77–85 parallels the reduction in the capacity to bind to the SRE.

Mutation of Ser101 to alanine (SRF-6His-M5), which reduced the degree of phosphorylation of Ser103, or replacing Ser103 itself with alanine (SRF-6His-M6) led to

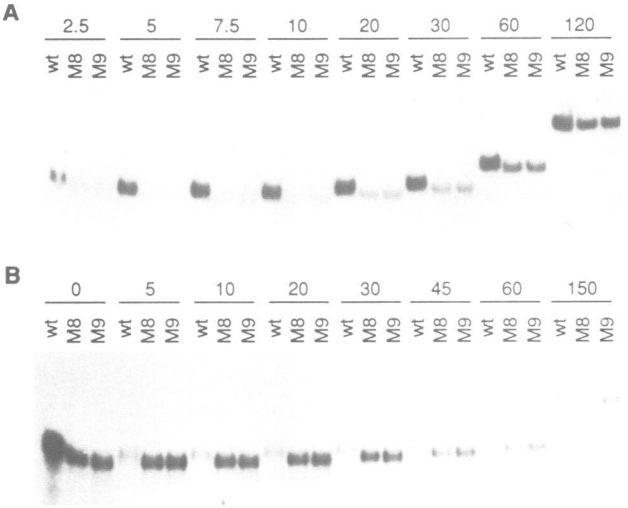


Fig. 8. DNA binding kinetics of SRF-6His mutants M8 and M9. (**A**) and (**B**) are analogous to Figure 7A and B, except that a larger excess of unlabelled competitor (40-fold) was used.

Table I. DNA binding properties of SRF-6His mutants

Protein	Relative DNA-binding activity[a]	On/off rates of DNA-binding
SRF-6His	100%	wild-type
SRF-6His-M1	88%	≈ wild-type
SRF-6His-M2	38%	decreased
SRF-6His-M3	29%	decreased
SRF-6His-M4	25%	decreased
SRF-6His-M5	23%	≈ wild-type
SRF-6His-M6	28%	≈ wild-type
SRF-6His-M8	52%	decreased
SRF-6His-M9	60%	decreased

[a]Relative DNA binding activity was calculated as follows: the amount of SRE complexed in a band shift assay, i.e. the 0 min time point of the dissociation kinetics shown in Figure 7B or 8B, was quantitated by Cerenkov counting and normalized for the relative concentration of the SRF-6His variant in the used protein extract. This relative protein concentration was measured by laser-densitometry of a corresponding Western blot (not shown).

an ~4-fold reduction in DNA binding activity (Table I). Interestingly, no drastic change in the kinetics of DNA binding was observed when compared with SRF-6His (Figure 7). In contrast to SRF-6His mutants M1−M4, the mutants M5 and M6 show only a partial correlation between the phosphorylation state at position 103 and DNA binding.

Combinations of SRF-6His-M4 with M5 (yielding mutant M8) and SRF-6His-M4 with M6 (yielding mutant M9) led to molecules that lack serines in the region 77−85 and additionally at either position 101 or 103. Both mutants showed markedly reduced on- and off-rates as compared with SRF-6His, as well as an ~2-fold reduced DNA binding activity under equilibrium conditions (Figure 8 and Table I). In the latter respect SRF-6His-M8 and M9 were, however, not as severely affected as SRF-6His-M4, M5 or M6.

Our findings can be summarized as follows: (i) phosphorylation at position 77/79 has little effect on DNA binding properties; (ii) phosphorylation at Ser83/85 and at Ser103 coordinately determines the affinity for the SRE; and (iii) phosphorylation at Ser83/85, but not at Ser103, significantly influences the kinetics of DNA binding.

Interaction with p62^{TCF} and in vitro transcriptional activity are not affected by SRF phosphoserine mutations

SRF and p62^{TCF} interact with each other to form a ternary complex with the SRE. This interaction seems to be important for full activity of the c-*fos* promoter *in vivo* (Shaw *et al.*, 1989b; Graham and Gilman, 1991). Therefore, we addressed the question whether phosphorylation in the N-terminal region of SRF might influence the interaction with p62^{TCF}. Figure 9A clearly shows that the SRF-6His mutants M1−M6 and M8−M9 were all capable of interacting with p62^{TCF} to form complex II, which is composed of the SRE, a dimer of the respective SRF-6His mutant and of p62^{TCF}. Since no significant differences were observed in the extent of complex II formation, phosphorylation in the N-terminal region of SRF apparently does not interfere with binding to p62^{TCF}.

We then tested our mutants for their effect on *in vitro* transcription. A template containing the c-*fos* SRE linked to the c-*fos* TATA-box was incubated overnight with a vast excess of SRF-6His or mutants thereof to guarantee that each SRE was occupied by these proteins. An aliquot was then added to HeLa nuclear extract depleted of endogenous SRF and transcription reactions were started. All mutants and SRF-6His displayed a comparable stimulation of *in vitro* transcription of the SRE-driven template (SRE-TATA, Figure 9B), while transcription from control promoter constructs containing no SRE was unaffected (NFI-TATA and MLP, Figure 9B). These data indicate that the transactivation function of SRF is, at least *in vitro*, not adversely influenced by mutating the N-terminal phosphorylation sites.

Discussion

The vaccinia virus expression system as a tool for studying phosphorylation

The determination of *in vivo* protein phosphorylation sites is often hampered by the scarcity of pure protein and also by the inefficient incorporation of ^{32}P into proteins during *in vivo* labelling. Expressing proteins with the vaccinia virus (Moss, 1991) can overcome these problems: high levels of protein are expressed, e.g. 5−10 μg of SRF-6His per 9 cm dish of HeLa cells, and high levels of ^{32}P incorporation are achieved with a relatively low input of radioactivity. For SRF-6His, this was ~5 × 10^6 c.p.m. per 9 cm dish of HeLa cells. Furthermore, the addition of a histidine tag to the protein allows the rapid purification of the fusion protein by affinity chromatography on Ni^{2+}-NTA agarose (Hochuli *et al.*, 1988). The histidine tag on SRF-6His does not alter functional properties of SRF, including nuclear translocation, DNA binding, *in vitro* transcriptional stimulation and interaction with p62^{TCF} (Janknecht *et al.*, 1991). Since phosphorylation is a necessary prerequisite for the function of SRF (Prywes *et al.*, 1988), this implies that SRF-6His is authentically phosphorylated. Consistent with this, we detected no difference in the phosphorylation pattern of the N-terminal CNBr fragment of tk-SRF-6His and SRF-6His in transfected and in vaccinia infected cells, respectively. These data suggest that SRF is phosphorylated in vaccinia

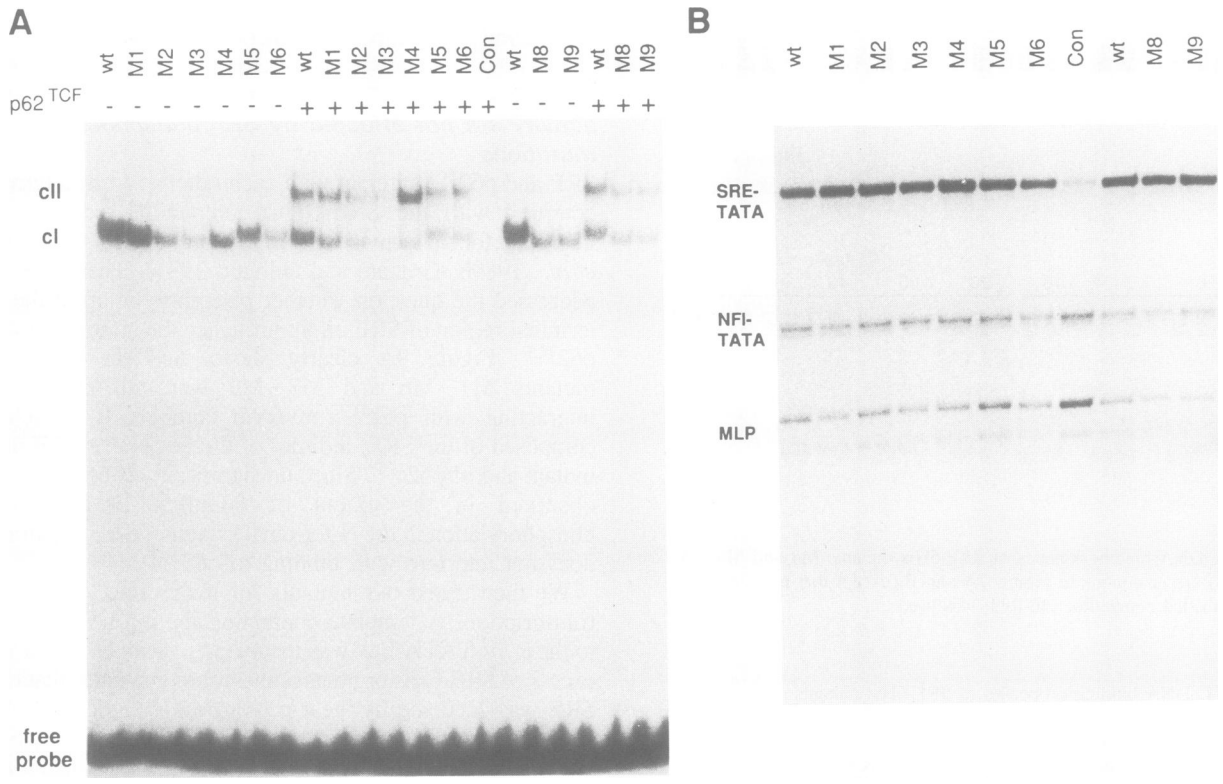


Fig. 9. Interaction of mutant derivatives of SRF-6His with $p62^{TCF}$ and *in vitro* transcriptional activity. (**A**) Protein extracts containing SRF-6His mutants were incubated in the presence or absence of $p62^{TCF}$ with labelled SRE for 30 min. Resulting complexes of SRF-6His mutants with the SRE (cI) and of SRF-6His mutants with both the SRE and $p62^{TCF}$ (cII) were resolved on a band shift gel and visualized by autoradiography. wt, SRF-6His; M1 to M6 and M8 to M9, SRF-6His mutants M1 to M6 and M8 to M9; Con, extract derived from HeLa cells infected with non-recombinant vaccinia virus. (**B**) SRE-TATA templates were saturated with a vast excess of the respective SRF-6His protein in an overnight binding reaction. An aliquot was then added to an *in vitro* transcription reaction assembled with SRF-depleted HeLa nuclear extract and two control G-free cassette templates driven by a nuclear factor I binding site fused to the c-*fos* TATA-box (NFI-TATA) or the adenovirus major late promoter (MLP). After a 60 min reaction at 30°C, transcripts were resolved on a sequence gel and visualized by autoradiography. Nomenclature is as in (A).

virus infected cells in the native pattern despite the presence of vaccinia virus encoded kinases or phosphatases (Guan *et al.*, 1991). Similar observations were also made with vaccinia expressed polyoma virus middle and large T-antigens (Guizani *et al.*, 1988) and c-Jun (J.Lou and H.G.Stunnenberg, unpublished results).

Phosphorylation sites on SRF affect its DNA binding properties

The vast majority of SRF phosphorylation (~85%) occurs in the N-terminal CNBr fragment, which encompasses 20% of the total protein. Five phosphorylation sites were mapped: a block of four serines at positions 77, 79, 83 and 85 and one phosphorylation site at Ser103. All the serines in the region 77–85 lie within a casein kinase II consensus recognition sequence ($S/TX_{0-2}E/D$; Kennelly and Krebs, 1991). Furthermore, casein kinase II can phosphorylate serines within amino acids 77–85 *in vitro* (Manak *et al.*, 1990; Manak and Prywes, 1991). Thus, it seems likely that casein kinase II is responsible for the phosphorylation at positions 77–85 *in vivo*. Casein kinase II or the cAMP-dependent protein kinase could be responsible for the phosphorylation at Ser103, as its surrounding amino acids ($KRSLS_{103}EME$) place it in both a casein kinase II and a less stringent cAMP-dependent protein kinase recognition sequence (RX_2S/T; Kennelly and Krebs, 1991). In particular, the sequence of amino acids 99–103 (KRSLS) is very similar to the sequence KRSGS found in phosphorylase kinase, where the last serine was shown to be phosphorylated by the cAMP-dependent protein kinase (Yeaman *et al.*, 1977).

Mutational analysis of SRF phosphorylation sites has shown that the degree of phosphorylation at positions 77–85 correlates with DNA binding. Furthermore, phosphorylation at Ser83 and 85 greatly influences the kinetics of DNA binding. Phosphorylation at Ser103 seems to be of comparable importance for DNA binding as the phosphorylation of all four serines at positions 77–85, because the decrease in DNA binding is roughly the same for SRF-6His mutants M4 and M6. But phosphorylation at Ser103 apparently exerts little effect on the kinetics of binding.

A striking observation is the effect of mutating the apparently unphosphorylated Ser101 to alanine in the mutants SRF-6His-M5 and M8. The DNA binding activity of these two mutants is reduced to slightly lower levels than in the corresponding Ser103 mutants. One possibility is that Ser101 plays an important role in protein conformation and accordingly influences DNA binding activity.

The SRF domain spanning amino acids 133–222 (SRF-core) is necessary and sufficient for binding to the SRE, albeit a SRF-core dimer binds more weakly than a SRF dimer (Norman *et al.*, 1988). Therefore, other regions of SRF must influence the strength of DNA binding. We have demonstrated that the N-terminal region is one of those regions, since all five phosphorylation sites in the region

77–103 affect DNA binding. One can envision that the phosphorylation state of the N-terminus influences the conformation of amino acids 133–222 and thereby affects the strength of SRF binding to the SRE.

Biological significance of the N-terminal phosphorylation

DNA binding activity is modulated via phosphorylation in a variety of transcription factors (Bohmann, 1990). By such a mechanism phosphorylation can directly determine transcriptional activity. This manner of transcriptional activation is unlikely for SRF, as the SRE seems to be constitutively occupied by SRF *in vivo* (Herrera *et al.*, 1989). Rather, SRF phosphorylation might influence c-*fos* transcription via the recruitment of SRF associated proteins necessary for transcriptional activation. We have shown that none of our mutations affect the interaction of SRF-6His with $p62^{TCF}$, thus eliminating a role for SRF N-terminal phosphorylation in complex formation with $p62^{TCF}$. It remains possible that other factors apart from $p62^{TCF}$ cooperate with SRF to regulate c-*fos* transcription and that these presumed interactions are dependent on the N-terminal phosphorylation of SRF.

If SRF bound to the SRE were by itself able to activate c-*fos* transcription, its N-terminal phosphorylation might determine transcriptional stimulation. As casein kinase II is likely to be responsible for the N-terminal phosphorylation of SRF, c-*fos* transcription would be linked to the activation of this kinase. This could explain the response of the c-*fos* gene to serum, epidermal growth factor or insulin, as casein kinase II is apparently induced by the same agents (Sommercorn *et al.*, 1987; Carroll and Marshak, 1989). Indeed, microinjection of casein kinase II led to phosphorylation of SRF and induction of the c-*fos* gene, with the latter being inhibited by coinjection of anti-SRF antibodies (Gauthier-Rouvière *et al.*, 1991). However, our *in vitro* transcription data show that mutations affecting N-terminal phosphorylation of SRF do not alter the transactivation function of SRF, albeit under conditions where transcription is not influenced by different DNA binding properties. Future experiments with those mutants should show whether the same holds true in a cellular context.

We imagine that our phosphorylation deficient mutants are functionally analogous to underphosphorylated SRF *in vivo*. Upon induction of quiescent cells newly synthesized SRF protein has an apparent lower molecular weight than 67 kDa (Misra *et al.*, 1991), which might reflect underphosphorylation. Accordingly, underphosphorylated species of SRF are produced after the induction of cells, migrate to the nucleus and could compete with fully phosphorylated SRF for binding to a variety of early gene promoters possessing a SRE. The exchange of fully phosphorylated by underphosphorylated SRF will take >30 min, because of two reasons: the delayed induction of the SRF gene in comparison with c-*fos* (Norman *et al.*, 1988) and the significantly lower association rate of underphosphorylated SRF that we have observed in this study. The exchange of fully phosphorylated by underphosphorylated SRF might have different effects depending on the promoter/SRE context: it may stimulate transcription thereby eliciting a late response or it may shut off an early response. The delayed kinetics of SRF activation make it unlikely that *de novo* synthesized SRF is mediating the activation of immediate early genes like c-*fos*, or the rapid repression of some immediate early genes that occurs within 30 min after induction. However, during the period when the c-*fos* promoter is refractory to induction (Morgan *et al.*, 1987; Büscher *et al.*, 1988), it is possible that underphosphorylated SRF molecules slowly replace fully phosphorylated SRF molecules on the c-*fos* SRE. Once this exchange is complete, the promoter might regain its capability to be induced. Our phosphorylation mutants will allow us to test these mechanisms *in vivo*, and thereby determine the role that phosphorylation in the SRF N-terminal region plays in the control of the c-*fos* gene.

Materials and methods

Purification of SRF-6His protein and its N-terminal CNBr fragment

Generation of SRF-6His recombinant vaccinia virus and infection of HeLa monolayer cells have been described previously (Janknecht *et al.*, 1991). Cells were incubated at 37°C in DMEM (Gibco) supplemented with 10% fetal calf serum for 5–6 h after infection. Then, the cells were incubated overnight in DMEM or in phosphate-free DMEM supplemented with 5% DMEM and 0.5 mCi/ml $^{32}P_i$ (Amersham). After the addition of 10% fetal calf serum the cells were incubated another 30 min, washed two times with PBS and then lysed in 0.1 M NaP_i, 6 M guanidine–HCl pH 8.0 for 5 min. The lysate derived from one 9 cm dish of HeLa cells labelled with $^{32}P_i$ was mixed with 10-fold the amount of non-radioactive lysate and then incubated with 0.5 ml Ni^{2+}-NTA–agarose (Qiagen) for 2–3 h under continuous tumbling. The resin was packed into a column and washed with 0.1 M NaP_i, 6 M guanidine–HCl pH 8.0, followed by the same buffer at pH 5.9. Bound proteins were eluted with 0.1 M NaP_i, 6 M guanidine–HCl pH 4.0 (Hochuli *et al.*, 1988), reduced with 10 mM DTT at 37°C overnight and then reacted with 40 mM 4-vinylpyridine at 20°C for 2 h. Then, the eluate was immediately chromatographed on a reverse phase column (Pro-RPC HR5/2, Pharmacia) using the FPLC system (Pharmacia). The column was developed with a gradient of acetonitrile in 0.1% trifluoroacetic acid. SRF-6His protein eluted at 31–33% acetonitrile and was lyophilized. The lyophilisate was dissolved in 70% formic acid, 50 mg/ml CNBr and digested for 24 h in the dark at 20°C. After lyophilization, peptides were dissolved in 0.1 M NaP_i, 6 M guanidine–HCl pH 8.0. Subsequent Ni^{2+}-NTA affinity chromatography and purification of the pH 4.0 eluate by reverse phase chromatography (Pep-RPC HR5/5 column, Pharmacia) were as described above. The N-terminal CNBr fragment eluted at 30–33% acetonitrile.

Phosphoamino acid analysis

The lyophilized N-terminal CNBr fragment (10 000 c.p.m.) of SRF-6His was cleaved with 6 M HCl in a low pressure N_2 atmosphere for 2 h at 110°C. Amino acids were separated by two-dimensional thin-layer electrophoresis (Cooper *et al.*, 1983). Phosphoamino acid markers were co-electrophoresed and identified by ninhydrin staining.

Cleavage of the N-terminal CNBr fragment

Digestions with chymotrypsin, trypsin or Glu-C were according to the manufacturer (Boehringer, Mannheim). Partial cleavage with 5% formic acid was performed at 106°C for 4 h in a low pressure N_2 atmosphere (Matsudaira, 1989). The resulting peptides were purified on a Pep-RPC HR5/5 column as described above.

Peptide sequencing

Automated sequencing of peptides was performed on an Applied Biosystems 477A pulsed-liquid protein sequencer using the standard sequencing program and the reagents provided by the manufacturer. The phenylthiohydantoin derivatives of amino acids liberated after each degradation cycle were identified and quantified by an on-line Applied Biosystems 120A HPLC system. Conversion of phosphoserine to S-ethylcysteine residues (Meyer *et al.*, 1986) was performed prior to sequencing.

Generation of mutant SRF-6His vaccinia viruses

Mutations were introduced into the *Sac*II–*Stu*I fragment of SRF cDNA (positions 570–870) using mutated oligonucleotides and standard polymerase chain reaction (PCR) techniques (Sambrook *et al.*, 1989). PCR generated DNA fragments were recloned into *Sac*II and *Stu*I restricted pBS-SRF, which consists of the *Eco*RI–*Bam*HI fragment of pSRF-6His (Janknecht *et al.*, 1991) inserted into pBS (Stratagene). PCR derived inserts were confirmed by DNA sequencing. The *Eco*RI–*Bam*HI fragments of mutated variants

of pBS-SRF were then cloned into pSRF-6His/*Eco*RI−*Bam*HI. Mutated variants of pSRF-6His were used to generate recombinant vaccinia viruses as described (Janknecht *et al.*, 1991).

Preparation of protein extracts

HeLa monolayer cells (one 9 cm dish) were infected with recombinant vaccinia virus. After 18 h cells were washed and then detached from the plate by incubation in 40 mM Tris−HCl, 10 mM EDTA, 150 mM NaCl pH 7.5 for 5 min. Cells were collected by centrifugation and the cell pellet was suspended in 0.5 ml of 50 mM Tris−HCl, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM NaF, 0.5% Triton X-100, 0.05% sodium deoxycholate, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride pH 7.5. After 20 min on ice debris were removed by centrifugation and the supernatant was frozen in liquid N_2 and stored at −70°C.

Protein gels and Western blotting

SDS−PAGE and Western blotting were performed according to standard procedures (Harlow and Lane, 1988). Rabbit anti-SRF antiserum raised against a C-terminal peptide was utilized for Western blots of protein extracts. Second antibodies were goat anti-rabbit antibodies conjugated with horseradish peroxidase. The immune complexes were visualized with an enhanced chemiluminescence Western blotting detection kit (Amersham).

Transfection of HeLa cells

SRF-6His was cloned into pEVRF0 (Matthias *et al.*, 1989), which fused the sequence MASWGSGTGPPLE to the N-terminus of SRF-6His. Thirty μg of this DNA construct was used to transfect HeLa cells grown to 50% confluency on a 9 cm dish by the calcium phosphate coprecipitation method (Sambrook *et al.*, 1989). The precipitate was removed after 6 h and the cells were grown in DMEM plus 10% fetal calf serum for 12 h. For starvation the medium was then changed to DMEM and incubation was continued for 8 h. Medium was then changed to phosphate-free DMEM supplemented with 5% normal DMEM and 0.5 mCi/ml $^{32}P_i$. For non-starved cells, 10% serum was included. 12 h later cells were induced for 25 min with 10% serum or directly lysed as described for vaccinia infected cells.

Band shift and in vitro transcription assays

Band shift assays were performed essentially as described (Runkel *et al.*, 1991). The ^{32}P-end-labelled SRE probe spanned the c-*fos* promoter from −330 to −278. *In vitro* transcription assays were performed according to Hipskind and Nordheim (1991).

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