Casein kinase II induces *c-fos* expression via the serum response element pathway and p67^{SRF} phosphorylation in living fibroblasts

Cécile Gauthier-Rouvière, Michèle Basset, Jean-Marie Blanchard¹, Jean-Claude Cavadore, Anne Fernandez and Ned J.C.Lamb²

Cell Biology, CRBM, CNRS/INSERM, BP 5051, 34033 Montpellier Cedex and ¹Laboratoire de Biologie Moléculaire, URA CNRS 1191, 34095 Montpellier Cedex, France

²Corresponding author

Communicated by I.W.Mattaj

Elevation of intracellular casein kinase II (CKII) levels through microinjection of purified CKII results in the rapid and transient induction of *c-fos* in quiescent rat embryo fibroblasts, and activation of quiescent cells by serum is accompanied by the nuclear relocation of endogenous CKII. The induction of *c-fos* by CKII is inhibited by coinjection of oligonucleotides corresponding to the sequence of the serum response element (SRE) present in the *c-fos* promoter, indicating that competitive displacement of positive factors from the endogenous *c-fos* SRE prevents *c-fos* induction by CKII. Furthermore, the expression of *c-fos* induced by either CKII injection or serum activation is also inhibited by microinjection of antibodies against the 67 kDa serum response factor (p67^{SRF}) indicating the absolute requirement of p67^{SRF} in this process. Finally, we show the specific phosphorylation of p67^{SRF} *in vivo* following microinjection of CKII into quiescent cells. Together, these data strongly support that CKII induces *c-fos* expression through binding/activation of the phosphorylated p67^{SRF} at the SRE sequence.

Key words: casein kinase II/*c-fos*/p67^{SRF}/SRE

Introduction

Transcription of the *c-fos* proto-oncogene, a member of the cellular 'immediate-early' gene family, is induced by serum and several growth factors (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984; Curran, 1988). This induction is rapid, transient and can occur in the absence of new protein synthesis suggesting that post-translational modifications are involved in its transcriptional regulation (Cochran *et al.*, 1984; Greenberg *et al.*, 1986). The *c-fos* enhancer contains a sequence element, the serum response element (SRE) located 300 bp upstream of the transcription start site, which is required for *c-fos* expression by whole serum, purified growth factors (Treisman, 1985, 1986; Gilman *et al.*, 1986; Fisch *et al.*, 1987; Greenberg *et al.*, 1987; Buscher *et al.*, 1988; Gilman, 1988; Sheng *et al.*, 1988; Stumpo *et al.*, 1988) and *ras* oncogene (Gauthier-Rouvière *et al.*, 1990). The SRE is a binding site for a protein called serum response factor (SRF or p67^{SRF}) (Gilman *et al.*, 1986; Treisman, 1986, 1987; Prywes and

Roeder, 1987; Schröter *et al.*, 1987) and another protein p62^{TCF}, has been described binding in a SRF-dependent manner to SRE (Herrera *et al.*, 1989; Shaw *et al.*, 1989; Schröter *et al.*, 1990). p67^{SRF} has been shown to be phosphorylated *in vivo* on serine residues, and phosphatase treatment reveals the requirement for phosphorylation for p67^{SRF}-DNA binding activity *in vitro* (Prywes *et al.*, 1988). Recently, Manack *et al.*, 1990 have shown that p67^{SRF} is specifically phosphorylated *in vitro* by CKII, a protein kinase which shows a rapidly stimulated activity following serum and growth factor addition (Sommercorn *et al.*, 1987; Klarlund and Czech, 1988; Ackerman and Osherooff, 1989; Carroll and Marshak, 1989). CKII is a ubiquitous serine/threonine kinase which has been located in both cytosol and nucleus of cells (for a review see Krebs *et al.*, 1988). CKII stimulation seems to be linked with the transitions from G₀ to G₁, from G₁ to S and during the transition between prophase and metaphase of meiotic cell division in *Xenopus laevis* oocytes (Mulner-Lorillon *et al.*, 1988). Interestingly, the transient pulse in CKII activity following cell activation is concomitant with transient rises in *c-fos* expression (Greenberg and Ziff, 1984) leading to the suggestion that CKII is directly upstream of p67^{SRF} in the signal transduction pathway that leads to *c-fos* transcriptional activation in response to growth factors.

In this report, we describe the rapid induction of *c-fos* expression following microinjection of purified CKII into quiescent REF-52 cells. We find that this CKII induced *c-fos* expression acts through a pathway utilizing SRE sequence and p67^{SRF} protein. Moreover, we observed *in vivo* phosphorylation of p67^{SRF} following microinjection of CKII, suggesting a direct link between *c-fos* induction by CKII and p67^{SRF} phosphorylation.

Results

Microinjection of purified casein kinase II into REF-52 cells rapidly induces *c-fos* expression

We initially examined whether quiescent cells would induce *c-fos* expression after microinjection of purified CKII. Whilst quiescent cells injected with only marker antibodies (Figure 1A) showed no *c-fos* synthesis (Figure 1B; consistent with the opinion that the microinjection technique does not artefactually induce *c-fos*), cells injected with CKII (from 10–50 µg/ml in the needle) (labelled with antibodies, Figure 1C) showed detectable *c-fos* staining as early as 30 min after injection (Figure 1D). This effect peaked by 60 min after injection (Figure 1E and F). At this peak of induction, 100% of the injected cells (50–100 cells/experiment) showed bright homogeneous nuclear fluorescence for *c-fos* staining. With longer incubation periods after injection, the extent of nuclear *c-fos* staining gradually declined from cells, until it was back to background level by 3–4 h post injection (data not shown). This CKII induced *c-fos* expression differs from

Fig. 1. Induction of *c-fos* in cells injected with purified CKII and nuclear localization of endogenous CKII following serum activation. To investigate if CKII induced *c-fos* expression, quiescent REF-52 cells plated on glass coverslips were microinjected with purified CKII (50–100 $\mu\text{g}/\text{ml}$; specific activity = 1.1 U/min/mg). At various times afterwards, the cells were fixed and stained for *c-fos* and marker antibody. Panels A and B: cells microinjected with control antibodies and stained for either the injected antibodies (panel A) or *c-fos* (panel B) 60 min after injection. Panels C–F: cells microinjected with control antibodies and purified CKII, and stained for either the injected antibodies (panels C and E) or *c-fos* (panels D and F) 30 min (panels C and D) or 60 min (panels E and F) after injection. Panels G–I: staining for the endogenous CKII using a rabbit anti-CKII antiserum against a CKII peptide as immunogen (see Materials and methods for the sequence of the peptide). Serum starved cells (panel G) and 60 min serum re-fed cells (panel H) were stained for CKII protein. In panel I, re-fed cells were stained with anti-CKII after saturation with the CKII peptide (1 mg/ml). Bar = 10 μm .

the serum-induced expression of *c-fos* in REF-52 cells in its rapidity and its intensity. Indeed, as described elsewhere (Lamb *et al.*, 1990) *c-fos* starts to be expressed 60 min after serum refeeding of REF-52 cells and peaks at 110–120 min after serum addition. (More recently, using a different batch

of serum, we observed the induction of *c-fos* as early as 30 min after serum addition peaking at 90 min.) With respect to such cells, the levels of *c-fos* following CKII injection is 2–3 times higher than that observed after serum addition (as determined by comparing exposure times on *c-fos*-stained

Fig. 2. Inhibition of CKII-induced *c-fos* expression by injection of SRE oligonucleotides. In order to assess if *c-fos* induction by CKII required the involvement of SRE, quiescent REF-52 cells plated on glass coverslips were microinjected with purified CKII and double-stranded DNA oligonucleotides corresponding to the SRE or the mutated SRE sequence (see Materials and methods). One hour afterwards, cells were fixed and stained for *c-fos* proteins and for marker injected antibodies. Panels A and B: cells microinjected with control antibodies, CKII and the mSRE oligonucleotides. Panels C and D: cells injected with control antibodies, CKII and the SRE oligonucleotides and stained for either the injected antibodies (panels A and C) or *c-fos* proteins (panels B and D).

immunofluorescent nuclei or densitometric scanning of negatives). This *c-fos* expression induced by CKII was dependent upon the presence of active casein kinase II since boiling or freeze-thaw inactivation of the enzyme resulted in an abolition of *c-fos* induction which was concomitant with the loss of *in vitro* CKII activity. Moreover, the same effect has been observed in a number of other cell lines (including NIH3T3, Swiss 3T3 and HS68).

To investigate the possible relevance of CKII in the induction of *c-fos*, (particularly following serum stimulation of quiescent cells), we examined the distribution of CKII during the transition from G_0 to G_1 . Localization of CKII in intact cells was studied by immunofluorescence using a rabbit anti-CKII antibody directed and affinity purified against the immune CKII peptide. Figure 1(G,H) shows that, in serum-starved cells, the enzyme was spread throughout the cell (G). In contrast, in refed cells, CKII was detected mainly in association with the nuclei (H). As a control, in Figure 1I the anti-CKII antiserum was first incubated with the immune peptide which resulted in the abolition of the nuclear staining in serum re-fed cells. These observations strongly suggest that the enzyme undergoes preferential recruiting in the nucleus concomitant with proliferative activation. A similar process has been hypothesized previously in bovine adrenocortical cells (Filhol *et al.*, 1990).

***c-fos* expression induced by casein kinase II is prevented by microinjection of SRE oligonucleotides**

Many of the proliferative pathways which induce *c-fos* expression have been shown to involve the SRE sequence (Treisman, 1985, 1987; Gilman *et al.*, 1986, 1988; Gauthier-Rouvière *et al.*, 1990; Lamb *et al.*, 1990). If CKII is to form part of the pathway of serum induced *c-fos* expression, it should involve the binding/activation of factors to the SRE region. To examine this possibility we have coinjected SRE oligonucleotides or a mutated control sequence (mSRE) (0.5 mg/ml) with purified CKII and examined the levels of *c-fos* expression. Whilst cells microinjected with mutated oligonucleotides induced *c-fos* expression with kinetics similar to those microinjected with CKII alone (Figure 2A and B), we observed little or no *c-fos* expression in cells microinjected with CKII in the presence of SRE sequences (Figure 2C and D). Ninety percent of the cells coinjected with CKII and SRE oligonucleotides did not express *c-fos* whilst 100% of the cells coinjected with CKII and mutated SRE showed intense *c-fos* expression (each experiment involved the injection of 30–50 cells and was repeated at least three times). This effect could be observed over a range of concentrations of SRE from 0.3–1 mg/ml whereas up to 2 mg/ml mutated SRE had no such effect (data not shown). This inhibition of CKII induced *c-fos* by coinjected SRE,

did not reflect only a delay in induction since we did not observe *c-fos* expression for up to 8 h following microinjection.

In addition, we have observed a similar CKII activity *in vitro* in the presence or absence of DNA SRE oligonucleotides, discounting that these oligonucleotides might directly affect the kinase activity when coinjected into cells (Table I). Furthermore, unlike the case with SRE, when an oligonucleotide corresponding to the TPA-response element (TRE; see sequence in Materials and methods), another

Table I. Effect of SRE oligonucleotides on CKII activity *in vitro*

Reaction	^{32}P (pmol/min) ^a
Complete	10.3
–Kinase	0.5
+SRE ^b	10.2
+mSRE ^b	10.1

^aCKII activity determined by the incorporation of ^{32}P into the dephosphorylated casein as described by Hathaway and Traugh (1983).

^bAssay performed in the presence of up to 0.3 mg/ml of DNA oligonucleotides (SRE or mSRE).

regulatory site present in the 5' non-coding region of the *c-fos* gene, was included in the microinjection buffer together with CKII, we observed no reduction in the levels of *c-fos* staining (data not shown), implying that displacing eventual protein factors from the TRE element does not affect *c-fos* induction by CKII in contrast to the effect observed by injecting SRE oligonucleotides (data not shown). This result further supports the specificity of the effect obtained with SRE injection.

***c-fos* expression is prevented by microinjection of anti-p67^{SRF} antibodies**

Previous reports have detailed that p67^{SRF} is phosphorylated *in vivo* on serine residues, and that phosphorylation is required for DNA binding activity *in vitro* (Prywes *et al.*, 1988). Since more recent examination *in vitro* has suggested a role for CKII in the phosphorylation of p67^{SRF} (Manak *et al.*, 1990), we examined if microinjection of anti-p67^{SRF} antibodies prevents *c-fos* expression by CKII.

For these experiments, we raised a polyclonal mouse serum against a 13 amino acid synthetic peptide (sequence derived from Prywes *et al.*, 1988) of p67^{SRF}. By immuno-

Fig. 3. *In vivo* p67^{SRF} turnover. In order to assess the time necessary to deplete the endogenous nuclear p67^{SRF}, growing REF-52 cells plated on glass coverslips were microinjected with rabbit anti-p67^{SRF} antibodies. At different times afterwards, the cells were fixed and stained for the distribution of p67^{SRF} and for injected antibodies. Cells microinjected with rabbit anti-p67^{SRF} antibodies and stained for either the injected antibodies (panels A, C and E) or p67^{SRF} proteins (panels B, D and F) 4 h (panels A and B), 7 h (panels C and D) or 12 h (panels E and F) after injection.

blotting, this serum recognized a protein of 67 kDa and by immunofluorescence gave a specific nuclear staining throughout the cycle and in quiescent cells. This antibody also immunoprecipitates a protein of molecular weight 67 kDa (Gauthier-Rouvière *et al.*, 1991).

Since we have observed the constitutive presence of p67^{SRF} in the nuclei of growing or quiescent cells (Gauthier-Rouvière *et al.*, 1991) and anti-p67^{SRF} antibodies do not freely pass into the nucleus when injected into the cytoplasm, we have performed initial experiments to assess the time necessary after anti-p67^{SRF} injection to deplete, through binding newly synthesized p67^{SRF}, the endogenous pool of p67^{SRF}. For these experiments, REF-52 cells were injected with rabbit antibodies directed against p67^{SRF} and subsequently fixed at various times and stained for the presence and distribution of p67^{SRF} with a mouse antiserum against p67^{SRF}. As shown in Figure 3, whilst 4 h after anti-p67^{SRF} injection, the staining for p67^{SRF} shows a nuclear distribution still intact and identical to the staining in non-injected surrounding cells (Figure 3, panels A and B), at

7 h post-injection, the intensity of p67^{SRF} staining has decreased as compared to that in non-injected cells (Figure 3, panels C and D) and by 12 h after injection of the anti-p67^{SRF} antibodies, p67^{SRF} staining is no longer discernible in the nuclei of injected cells (Figure 3, panels E and F). Although p67^{SRF} is a nuclear protein, this result suggests that its neutralization by the injected antibodies can effectively occur in the cytoplasm. Indeed, we would predict this antisera to bind to p67^{SRF}, in light of its ability to induce changes in electrophoretic mobility (as assessed by mobility shift assay; Gauthier-Rouvière *et al.*, 1991) even though the epitope is outside the DNA binding domain. This experiment allows us to conclude that the turnover of p67^{SRF} *in vivo* is ~10–12 h. In addition, a recent review has reported a similar half-life for p67^{SRF} in mouse 3T3 fibroblasts (Rivera and Greenberg, 1990). Therefore, to allow the injected antibodies to neutralize newly synthesized p67^{SRF} in the cytoplasm, and ensure that the p67^{SRF} present in the nuclei had turned over, injections of antibodies against p67^{SRF} were performed 10–12 h

Fig. 4. Inhibition by anti-p67^{SRF} of c-fos induced by serum. To assess if mouse anti-p67^{SRF} antibodies inhibit c-fos induced by serum, quiescent REF-52 cells plated on glass coverslips were microinjected 10 h before serum refeeding with either control mouse serum or mouse anti-p67^{SRF}. 90 min after serum refeeding, the cells were fixed and stained for c-fos and marker antibodies. Panels A and B: cells microinjected with control mouse serum; panels C and D: cells microinjected with mouse anti-p67^{SRF} antibodies into the cytoplasm only; panels E and F: cells microinjected with mouse p67^{SRF} antibodies into the cytoplasm and the nucleus. Cells were stained for the injected antibodies (panels A and C) and for c-fos proteins (panels C and D).

before the induction of *c-fos* through serum addition or CKII microinjection.

Our initial experiments examined if the mouse anti-p67^{SRF} antibodies could modulate the induction of *c-fos* expression by serum (Figure 4). Whilst in cells injected with control mouse antibodies (Figure 4A), *c-fos* induction occurred (Figure 4B) in a manner similar to surrounding non-injected cells (i.e. 100% of cells at 90 min), the injection of anti-p67^{SRF} antibodies (0.25–0.5 mg/ml) (Figure 4C) was clearly effective in preventing the induction of *c-fos* by serum (Figure 4D) (90–100% of inhibition). Alternatively nuclear microinjection could allow a more immediate binding of the anti-p67^{SRF} to p67^{SRF}, but since this type of microinjection always produces a transient stress response, an artefactual induction of *c-fos* through this technique could not be discounted if injection was performed shortly before monitoring for *c-fos* induction, therefore such nuclear injection was also performed several hours (5–6 h) before induction of *c-fos* by serum addition.

Likewise microinjection in the cytoplasm, microinjection of anti-p67^{SRF} in the nucleus (Figure 4E and F) resulted in an apparently equal inhibition of *c-fos* induced by serum.

In a similar series of experiments to those described above, we examined if these mouse anti-p67^{SRF} antibodies could similarly prevent induction of *c-fos* expression by CKII (Figure 5). We again injected control mouse serum (Figure 5A) or anti-p67^{SRF} serum (Figure 5C) into the cytoplasm of the cells 12 h before microinjection of CKII. To ensure effective double microinjection, cells were demarcated on the coverslip by creating a square formed by scraping

adjacent cells; subsequently all cells in this delineated region (50–60 cells) were microinjected 60 min after CKII injection, the cells were fixed and stained for *c-fos* expression (Figure 5B and D). Prior injection of mouse anti-p67^{SRF} antibodies effectively prevented CKII-induced *c-fos* (Figure 5D; 90–100% of inhibition), whereas prior injection of control serum had no effect on *c-fos* induction by CKII (Figure 5B).

This effect was dependent on active anti-p67^{SRF} since the inhibition of *c-fos* induction could be lost by prior treatment of the antibody with DTT, by passing the serum through an anti-mouse IgG column or by prior incubation of the antibody with the SRF immune peptide (data not shown).

Taken together, these results indicate that the induction of *c-fos* expression by CKII, likewise its induction by serum, involves SRE and the binding and/or activation of p67^{SRF}.

CKII microinjection induces phosphorylation of p67^{SRF}

In order to investigate further the action of CKII on p67^{SRF} *in vivo*, we performed metabolic labelling with [³²P]H₃PO₄ of quiescent G₀ cells or serum refeed cells and compared it with quiescent cells injected with CKII, to examine if we could discern changes in phosphorylation of p67^{SRF}.

To determine the position of p67^{SRF} unambiguously we performed simultaneous immunoblot analysis of the labelled cells after separation of protein by 2-D gel electrophoresis on a basic gradient from pH 5 to 8.5. As shown by O'Farrell (1975) such 2-D separation allows all proteins to be separated as individual spots without possible overlapping of different proteins. This can be further guaranteed by running the same

Fig. 5. Inhibition by anti-p67^{SRF} of *c-fos* induced by CKII. In order to examine if mouse anti-p67^{SRF} antibodies could prevent *c-fos* induction by CKII, quiescent REF-52 cells plated on glass coverslips were microinjected with anti-p67^{SRF}. 10 h later, the same cells were reinjected with purified CKII. One hour after CKII microinjection, cells were fixed and stained for *c-fos* and marker antibodies. Panels A and B, cells microinjected with control mouse serum 10 h before injection of CKII; panels C and D, cells microinjected with mouse anti-p67^{SRF} antibodies 10 h before injection of CKII and stained either for the injected antibodies (panels A and C) or *c-fos* (panels B and D).

Fig. 6. *In vivo* phosphorylation of p67^{SRF} by CKII. Changes in p67^{SRF} phosphorylation were investigated following microinjection of quiescent REF-52 cells plated on glass chips with CKII (50–100 µg/ml) (all of the cells, ~200, on the coverslip were injected). Alternatively, chips containing ~1000 quiescent cells were directly labelled either in the absence of serum or following addition of 8% serum. The cells were metabolically labelled with 0.25 mCi of [³²P]H₃PO₄ in 5 µl of phosphate free DMEM for 30 min. After the labelling period, the cells were briefly washed in PBS and the chips were transferred to Eppendorf tubes containing ~5 × 10⁶ unlabelled cells (as carrier proteins) in lysis/sample buffer and analysed by 2-D electrophoresis as described in Materials and methods. To determine exactly the position of p67^{SRF} into the pattern of proteins analyzed by 2-D electrophoresis we performed 2-D immunoblot with two different anti-p67^{SRF} antibodies. We also performed immunoprecipitation using nuclear extracts from cells prelabelled with [³⁵S]methionine and with the same two anti-p67^{SRF} antibodies. The regions of the immunoblot and of the autoradiograms covering the area of migration of p67^{SRF} are shown. Panel A: Immunoblot of p67^{SRF} from serum refed cold cells as revealed with anti-p67^{SRF} (added as carrier proteins to the CKII injected cells). Panel B: p67^{SRF} immunoprecipitated from randomly growing cells with anti-p67^{SRF}. Panel C: Immunoblot of p67^{SRF} as revealed from serum starved cells with anti-p67^{SRF} (blot from autoradiogram shown in E). Panel D: p67^{SRF} immunoprecipitated from randomly growing cells with anti-p67^{SRF}. Panel E: Autoradiogram from 2-D blot analysis of quiescent cells labelled from 0 to 45 min (1000 cells labelled). Panel F: Autoradiogram from 2-D blot analysis of quiescent cells after addition of 8% fetal calf serum and labelled from 0 to 45 min (1000 cells labelled). Panel G: Autoradiogram from 2-D blot analysis shown in panel A, corresponding to 200 quiescent cells injected with purified CKII and labelled from 15 to 45 min.

sample with two different pH gradients (which we have done for p67^{SRF} identification). Using two different peptide antibodies (anti-p67^{SRF1} and anti-p67^{SRF2}) we performed immunoblot (Figure 6, panels A,C) as well as immunoprecipitation analysis (Figure 6, panels B,D). As shown in Figure 6 panels A, B, C and D, p67^{SRF} is detected as a basic protein containing several isoforms (pHi around 8). Comparison of the Western blot staining in panel A, which represent serum refed cells (added as cold carrier to the CKII injected cells, autoradiogram shown in panel G), to the staining in panel C which correspond to serum starved cells (autoradiogram of this blot is shown in panel E), shows that the relative abundance of these isoforms varies in correlation with their phosphorylation status. Panel A shows two major isoforms with the more acidic one being relatively abundant and the presence of a third most acidic but minor isoform can be detected on this blot. This pattern correlates well with the ³²P spot distribution seen in panel G. In contrast, panel C shows one major isoform, with a second more acidic one being barely detectable. Although the pattern of staining shown in panel C was obtained using a second antibody to p67^{SRF}, detection of p67^{SRF} in quiescent cells using anti-p67^{SRF1} gives a similar pattern to anti-p67^{SRF2} (data not shown). Figure 6E and F shows the phosphorylation status of p67^{SRF} in quiescent (E) and 45 min (F) refed cells. Whilst little or no phosphate associated with p67^{SRF} isoforms can be resolved in quiescent cells (Figure 6E) (obtained from 2-D protein analysis of 1000 serum starved labelled cells), in refed cells (obtained from 1000 labelled cells), we observed a major increase in phosphorylation of the two isoforms (Figure 6F). Since there is ample evidence that p67^{SRF} is a phosphoprotein, phosphorylated *in vivo* on serine and by CKII *in vitro*, we examined if microinjection of purified CKII induced changes in p67^{SRF} phosphorylation *in vivo* and furthermore, if this was similar to that observed in the serum refed cells. In cells microinjected with CKII (200 microinjected labelled cells), we observed that p67^{SRF} become rapidly phosphorylated after 30 min (Figure 6G) (Note that this is the autoradiogram from the blot shown in Figure 6A). It is also important to notice that the increase in phosphorylation observed, associated with the immune stained p67^{SRF} spots in CKII injected or serum stimulated cells, does not reflect a general increase in phosphorylation of all proteins since other phosphoproteins around show comparable labelling intensity (note that the doublet above the most acidic form of p67^{SRF} is present in panels E, F and G). The finding that this phosphorylation did not occur in serum starved cells but appeared 45 min after serum treatment or CKII microinjection, strongly implied that the changes in p67^{SRF} activity observed following CKII treatment *in vitro* may also be involved in regulating p67^{SRF} activity *in vivo*. Moreover, the level of label incorporation observed in cells injected with CKII is not very different from the level observed in serum refed cells even though fewer cells were analysed (200/1000), implying that CKII can induce a strong phosphorylation signal of p67^{SRF}.

Reciprocally, we have observed a marked reduction in p67^{SRF} phosphorylation in serum refed cells following anti-CKII microinjection (data not shown). However, whilst this further supports the conclusion that a relationship exists between p67^{SRF} phosphorylation and CKII activity, anti-CKII antibodies injection was also accompanied by a pronounced decrease in overall protein phosphorylation. Together, these results strongly support the hypothesis that

CKII phosphorylates p67^{SRF} *in vivo* following its injection into rat fibroblasts, in agreement with a recent report showing that *in vitro* CKII-dependent phosphorylation of p67^{SRF} enhances its DNA binding activity (Manak *et al.*, 1990).

Discussion

Using microinjection of purified casein kinase II (CKII) in quiescent normal fibroblasts, we have examined the effect of elevating intracellular CKII levels on *c-fos* expression. Microinjection of purified active CKII into quiescent fibroblasts induces the rapid expression of *c-fos*. Such induction of *c-fos* expression by CKII is prevented by coinjection of double-stranded DNA oligonucleotides corresponding to the serum response element SRE or by antibodies directed against p67^{SRF}. In addition, we have observed that p67^{SRF} increases its phosphorylation *in vivo* following CKII injection suggesting that CKII-induced *c-fos* expression is mediated through p67^{SRF} phosphorylation by CKII.

Induction of *c-fos* expression by CKII

It is now well known that transcription of the *c-fos* proto-oncogene is rapidly induced by serum growth factors (Greenberg *et al.*, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984). Such factors acting through cell-surface receptors activate a variety of distinct signalling pathways within the cell (Rozengurt, 1986) which reach the nucleus and lead to changes in gene expression such as that of *c-fos*. *c-fos* induction can also occur by direct activation of distinct second messengers such as protein kinase C, calcium and cAMP (for a review, see Curran, 1988). Although CKII has been shown to be activated following growth factor stimulation (Sommercon *et al.*, 1987; Klarlund and Czech, 1988). EGF treatment of A₄₃₁ cells (Ackerman *et al.*, 1990) and concomitantly with a transient rise in *c-fos* expression (Carroll *et al.*, 1988) at the G₀ to G₁ transition, no direct link had been established before between CKII activation and *c-fos* induction. Our results show for the first time that microinjection of purified active CKII can, in the absence of any serum or growth factors, induce *c-fos* expression in quiescent fibroblasts. Interestingly, this induction of *c-fos* by CKII was both faster (maximum induction by 60 min) and more intense (as judged by comparative immunofluorescence staining) than the induction of *c-fos* by serum in the same cells. Using immunocytochemical localization, we also observed a preferential relocation of CKII within the nuclei of cells upon serum stimulation whereas in serum starved cells the enzyme seems to be spread throughout the cytoplasm. Together with the induction of *c-fos* expression by CKII, this observation strongly supports the notion of a potential role of this enzyme at the G₀ to G₁ transition and on *c-fos* expression after growth factor addition.

c-fos expression following serum or growth factors addition involves different regulatory sequences in the 5' flanking region of the *c-fos* gene (for a review see Verma and Sassone-Corsi, 1987). Among these, the serum response element SRE located 300 bp upstream of the *c-fos* mRNA start site has been shown to mediate the response to serum, EGF, phorbol esters and *ras* oncoprotein (Treisman, 1986; Gilman *et al.*, 1986; Fisch *et al.*, 1987; Gauthier-Rouvière *et al.*, 1990). Indeed, induction of *c-fos* by serum or *ras* can be specifically inhibited through microinjection of SRE

oligonucleotides which presumably compete for the binding of serum response factor protein(s) to the promoter of the gene (Gilman, 1988; Lamb *et al.*, 1990). In this respect, we found that CKII acts to induce *c-fos* in a manner similar to serum, since the addition of SRE oligonucleotides to microinjected CKII proteins effectively prevented *c-fos* induction by CKII. We showed that this inhibition is specific to SRE since injection of control oligonucleotides (mSRE) was ineffective in inhibiting *c-fos* expression by CKII.

Induction of *c-fos* expression by CKII or serum occurs via p67^{SRF}

Several groups have established a correlation between the binding of a 67 kDa serum response factor (p67^{SRF}) and SRE activity leading to the idea that p67^{SRF} at least in part mediates transcriptional induction by growth factors (Fisch *et al.*, 1987; Greenberg *et al.*, 1987; Treisman, 1987; Gilman, 1988).

In order to clarify further the role of p67^{SRF} in modulating SRE function, we produced a mouse antiserum against a synthetic peptide of 13 amino acids of p67^{SRF} (the sequence of which was derived from Prywes and Roeder, 1986). When injected long enough in advance to allow for the p67^{SRF} protein expression to turn over i.e. 9–10 h as shown in the present study (Figure 3) and also recently reported in a review by Rivera and Greenberg (1990), microinjection of this antibody was effective in preventing *c-fos* expression induced by serum, whereas a control serum had no effect on *c-fos* expression. Such a demonstration that neutralization of p67^{SRF} protein *in vivo* prevents *c-fos* expression implies that an activation of p67^{SRF} (binding and/or post-translational modifications) is required for *c-fos* induction in living cells. This result is not surprising in view of the previous evidence implicating p67^{SRF} as a positively acting transcription factor on the *c-fos* SRE (Norman *et al.*, 1988), but it showed that our anti-p67^{SRF} could be reliably used to neutralize p67^{SRF} *in vivo*. Since a similar blocking effect of anti-p67^{SRF} was obtained for induction of *c-fos* by CKII, it supports that CKII induces *c-fos* expression via an effect on p67^{SRF} phosphorylation. Together with the finding that CKII microinjection induces *c-fos* expression, this result strongly supports the idea that CKII is an integral part of the pathway(s) involved in serum induction of *c-fos*.

In vivo phosphorylation of p67^{SRF} by CKII

In order to investigate this possibility further, we undertook to examine if there were similarities between changes in p67^{SRF} phosphorylation following serum addition or CKII injection. Since (as shown in Figures 3 and 4), there are clearly very close similarities between serum and CKII, this would suggest that CKII or CKII-dependent pathways can be directly implicated in the induction of *c-fos*. A number of observations have shown that EGF treatment of quiescent cells leads to CKII activation (Ackerman *et al.*, 1990), which is concomitant with the G₀–G₁ transition (Carroll *et al.*, 1988) and that p67^{SRF} becomes phosphorylated *in vivo* on serine residues following EGF treatment (Prywes *et al.*, 1988). Such a phosphorylation, which *in vitro*, can be related to requirement for DNA binding activity (Prywes and Roeder, 1986; Prywes *et al.*, 1988). Following microinjection of CKII into quiescent fibroblasts, we found a specific and marked increase in p67^{SRF} phosphorylation, comparable to that observed in cells after growth factor

stimulation. In light of this result and since a recent report has shown that CKII phosphorylates p67^{SRF} *in vitro* on a site which is also found to be phosphorylated *in vivo* (Manak *et al.*, 1990), we would propose that the effective induction of p67^{SRF}-dependent *c-fos* expression by CKII relates directly to the CKII-dependent phosphorylation of p67^{SRF} *in vivo*. Since p67^{SRF} and p62 have been shown before to be permanently bound to SRE (before, during and after serum stimulation of quiescent normal cells) (Fisch *et al.*, 1987; Herrera *et al.*, 1989), and since we found that p67^{SRF} is constitutively present in the nuclei of cells at all stages of their division cycle (Gauthier-Rouvière *et al.*, 1991), one might speculate that such a phosphorylation of p67^{SRF} may act to directly modulate these interactions. Considering CKII as a good candidate for such p67^{SRF} activation, the interesting question remains, however, of which potential effector(s) might be involved in the activation of CKII, downstream of growth factor stimulation. In this respect, the protein kinase *c-raf* activation of which has been shown to involve a tyrosine phosphorylation (and as such might involve EGF) would represent a good candidate and we are currently investigating this possibility further.

Materials and methods

Cells

Rat embryo fibroblasts (REF-52) (McClure *et al.*, 1982) cells were cultured on 35 mm plastic dishes, glass coverslips or 2 mm glass chips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% fetal calf serum (FCS) in a humidified atmosphere containing 5% CO₂, 95% air as described elsewhere (Lamb *et al.*, 1988). Cells were plated 2–3 days before use.

Polyclonal antibodies production

BALB/c mice were initially injected subcutaneously with 100 µg of p67^{SRF} peptide–BSA in complete Freund's adjuvant (peptide sequence derived from Prywes and Roeder, 1986; ²⁹³TSPLFPITNYVAN³⁰⁵). After the first injection, animals were boosted every 2 weeks with 100 µg of peptide–BSA diluted in incomplete Freund's adjuvant and bled 3 days after each booster injection. Male New Zealand rabbits were injected with 300 µg of peptide–tyroglobulin as described elsewhere (Gauthier-Rouvière *et al.*, 1991) (two different peptides were used: ²⁹³TSPLFPITNYVAN³⁰⁵ and ⁹¹LGAEER-GLKRLSE¹⁰⁴). Anti-casein kinase II antibodies were obtained in rabbit using the following peptide: ⁵¹DLEPDEELED⁶⁰. The terminal cysteines were added on each peptide sequence for protein binding purpose. The production of anti-p67^{SRF} and anti-CKII antibodies was tested by immunofluorescence and immunoblotting.

Casein kinase II purification

Casein kinase II was purified from rabbit reticulocytes according to Hathaway and Traugh (1983). The resulting enzyme had high specific activity (1.1 units/mg protein) (one unit of enzyme activity equals 1 µmol ³²P incorporated into casein per minute) and the preparation was >99% pure as shown by silver staining of polyacrylamide gel.

Microinjection and metabolic labelling

Quiescent REF-52 cells plated on glass chips were microinjected with casein kinase (0.05–0.1 mg/ml). As controls quiescent cells also growing on chips were either serum refed or kept starved for simultaneous labelling. For labelling, the chips were transferred to humidified chambers, washed once in phosphate-free DMEM before incubation for 30 min periods in 5 µl of phosphate-free DMEM containing 0.25–0.5 mCi [³²P]H₂PO₄ plus or minus serum. After the end of the labelling period, each chip was washed with PBS and collected in 15 µl of lysis buffer (5% (wt/vol) β-mercaptoethanol, 1% (wt/vol) NP-40) and 15 µl of sample buffer (40 mM Tris–Cl (pH 6.8), 5 mM dithiothreitol, 1% (wt/vol) SDS, 7.5% (wt/vol) glycerol and 1% (wt/vol) bromophenol blue) in an Eppendorf tube containing 3 × 10⁶ pelleted cold REF-52 cells added as carrier protein. After boiling, samples were lyophilized and resuspended in 10 µl of 1 mg/ml DNase and 1 mg/ml RNase for 5 min at 37°C to digest nucleic acids. Samples were then diluted with IEF sample buffer (9.5 M urea, 2% (wt/vol) NP-40, 5% (wt/vol) β-mercaptoethanol, 2% (wt/vol) ampholines) such that the final

concentration of SDS was <0.3% (wt/vol) before analysis by 2-D gel electrophoresis and Western blotting.

For immunofluorescence experiments, REF-52 cells were plated on glass coverslips and microinjected with CKII in a solution containing marker antibodies (mouse Ig, 0.5 mg/ml) and either double-stranded DNA oligonucleotides corresponding to SRE (5'-AGGATGTCCATTAGGA-CATCTGC-3', mSRE (5'-AGGATGTCCATTAACTATTGATG-3') or TRE (5'-CATTAGCATGACTCATTGTTCT-3') (1 mg/ml in 100 mM HEPES).

In p67^{SRF} neutralization experiments, mouse anti-p67^{SRF} serum or control mouse serum was injected 10–12 h before serum refeeding or injection of CKII.

Immunofluorescence

At different times after microinjection, cells were fixed for 5 min in 3.7% formalin (in PBS) followed by a 30 s extraction in –20°C acetone and rehydration in PBS containing 0.1% BSA.

Injected antibodies and *c-fos* staining were visualized by a 60 min incubation with affinity-purified fluorescein-conjugated goat anti-mouse antibody diluted 1:100 (Cappel-Organon Technika) and a rabbit anti-*fos* antibody obtained using the same gag-*fos* fusion protein as Verrier *et al.* (1986) diluted 1:100 for 60 min. After a brief wash in PBS, the cells were incubated with biotinylated anti-rabbit antibody (Amersham) diluted 1:100 for 60 min and with streptavidin–Texas red (Amersham) diluted 1:500 for 30 min.

For the simultaneous detection of injected rabbit antibodies and p67^{SRF} proteins, fixed cells were incubated for 60 min with mouse anti-p67^{SRF} antibodies diluted 1:100 followed by a 60 min incubation with biotinylated anti-mouse antibody (Amersham) diluted 1:100 and by a 30 min incubation with streptavidin–Texas red diluted 1:500 and fluorescein-conjugated goat anti-rabbit antibodies diluted 1:100 (Cappel-Organon Technika).

Cells were washed in PBS, mounted in 0.25% (w/v) Airvol-205 in PBS and examined on a Zeiss Axiophot using ×63 (1.3 NA), ×40 or ×16 planapochromat lens. Fluorescent images were recorded on Kodak Tri-X pan film and processed as described before (Lamb *et al.*, 1988).

Immunoprecipitation on nuclear fractions

Three to four 60 mm dishes of growing REF-52 cells were labelled with [³⁵S]methionine (0.5 mCi/dish containing ~1–5 × 10⁶ cells) for 15 h. After this time, the cells were washed once with cold PBS and scraped into 1 ml of buffer A (10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.5% (wt/vol) NP-40). Cells were lysed with the Dounce homogenizer (B piston; 10-fold), placed in 1 ml buffer B (50 mM Tris–HCl (pH 8.3), 30% (wt/vol) sucrose, 5 mM MgCl₂, 0.1 mM EDTA) and spun for 5 min at 2000 r.p.m.. Pellet (nuclei) was resuspended in 50–100 µl of buffer C [50 mM Tris–HCl (pH 8.3), 40% (wt/vol) glycerol, 5 mM MgCl₂, 0.1 mM EDTA]. For immunoprecipitation experiments, four volumes of RIPA buffer [1% NP-40, 1% Na-deoxycholate, 0.4 M NaCl, 0.01 M PBS (pH 7.2), 0.1% SDS, 0.02% thimerosal] was added. p67^{SRF} was immunoprecipitated using successive incubations with anti-p67^{SRF} antibodies (2 h at 4°C) and protein A–Sepharose (1 h at 4°C). The precipitate was washed three times with TBS buffer (pH 7.3) containing 1% TX-100, 1% Na-deoxycholate, 0.1% SDS, 0.1% Na₃, resuspended in hot Laemmli sample buffer and analysed by 2-D gel electrophoresis as described below.

Two-dimensional gel electrophoresis and immunoblotting

Samples were analysed by 2-D electrophoresis using the method of O'Farrell (1975) as described before (Gauthier-Rouvière *et al.*, 1990) with the following changes. The ampholine mixture used contained 60% (wt/vol) ampholine pH 3–10, 40% (wt/vol) ampholine pH 5–7. Immediately after 2-D gel electrophoresis, proteins were electrophoretically transferred to nitrocellulose for 2 h at 70 V. On completion of the transfer, nitrocellulose sheets were incubated with 8% (wt/vol) powdered milk for 1 h to block free protein binding sites before being incubated with anti-p67^{SRF} antibodies diluted 1:100 for 1 h. Primary antibodies were visualized by incubation with biotinylated anti-rabbit antibodies (Amersham) diluted 1:200 for 1 h followed by streptavidin–horseradish peroxidase diluted 1:1000 for 30 min before subsequent colour development with 4-chloronaphthol (Sigma) [0.3% (w/v) in methanol] diluted 1:15 in PBS containing 20 µl H₂O₂.

The phosphoproteins are subsequently visualized by autoradiography of the blot on Kodak XAR-5 film at –70°C, using Cronex Lightening Plus Intensifying screens.

Acknowledgements

We are grateful to J.Demaille for his enthusiastic support for this work and to M.Piechaczyc for the gift of the gag-*fos* fusion protein. We would

like to thank the Association pour la Recherche contre le Cancer (contract no. 6140) and AFM for financial support.

References

- Ackerman,P. and Osheroﬀ,N. (1989) *J. Biol. Chem.*, **264**, 11958–11965.
- Ackerman,P., Glover,C.V.C. and Osheroﬀ,N. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 821–825.
- Buscher,M., Rahmsdorf,H.J., Litfin,M., Karin,M. and Herrlich,P. (1988) *Oncogene*, **3**, 301–311.
- Carrol,D. and Marshak,D.R. (1989) *J. Biol. Chem.*, **264**, 7345–7348.
- Cochran,B.H., Zullo,J., Verma,I.M. and Stiles,C.D. (1984) *Science*, **226**, 1080–1082.
- Curran,T. (1988) Reddy,E.P., Skalka,A.M. and Curran,T. (eds), *The Oncogene Handbook*. Elsevier, Amsterdam, pp. 307–326.
- Filhol,O., Cochet,C. and Chambaz,E.M. (1990) *Biochemistry*, **29**, 9928–9936.
- Fisch,T.M., Prywes,R. and Roeder,R.G. (1987) *Mol. Cell. Biol.*, **7**, 3490–3502.
- Gauthier-Rouvière,C., Fernandez,A. and Lamb,N.J.C. (1990) *EMBO J.*, **9**, 171–180.
- Gauthier-Rouvière,C., Cavadore,J.C., Blanchard,J.M., Lamb,N.J.C. and Fernandez,A. (1991) *Cell Regul.*, **2**, 575–588.
- Gilman,M.Z., Wilson,R.N. and Weinberg,R.A. (1986) *Mol. Cell. Biol.*, **6**, 4305–4316.
- Gilman,M.Z. (1988) *Genes Dev.*, **2**, 394–402.
- Greenberg,M.E. and Ziff,E.B. (1984) *Nature*, **311**, 433–438.
- Greenberg,M.E., Hermanowski,A.L. and Ziff,E.B. (1986) *Mol. Cell. Biol.*, **6**, 1050–1057.
- Greenberg,M.E., Siegfried,Z. and Ziff,E.B. (1987) *Mol. Cell. Biol.*, **7**, 1217–1225.
- Hathaway,G.M. and Traugh,J.H. (1983) *Methods Enzymol.*, **99**, 324–325.
- Herrera,R.E., Shaw,P.E. and Nordheim,A. (1989) *Nature*, **340**, 68–70.
- Klarlund,J.K. and Czech,M.P. (1988) *J. Biol. Chem.*, **263**, 15873–15875.
- Krebs,E.G., Eisenman,R.N., Kuenzel,E.A., Litchfield,D.W., Lozeman,F.J., Lüscher,B. and Sommercorn,J. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **LIII**, 77–84.
- Kruijer,W., Cooper,J.A., Hunter,T. and Verma,I.M. (1984) *Nature*, **312**, 711–716.
- Lamb,N.J.C., Fernandez,A., Conti,M.A., Adelstein,R., Glass,D.R., Welch,W.J. and Feramisco,J.R. (1988) *J. Cell Biol.*, **106**, 1955–1971.
- Lamb,N.J.C., Fernandez,A., Tourkine,N., Jeanteur,P. and Blanchard,J.M. (1990) *Cell*, **61**, 485–496.
- Manak,J.R., Bisschop,N., Kris,R.M. and Prywes,R. (1990) *Genes Dev.*, **4**, 955–967.
- McClure,D.B., Hightower,M.J. and Topp,W.C. (1982) *Cold Spring Harbor Conf. Cell Prolif.*, **9**, 345–364.
- Muller,R., Bravo,R., Burckhart,J. and Curran,T. (1984) *Nature*, **312**, 716–720.
- Mulner-Lorillon,O., Marot,J., Cayla,X., Pouhle,R. and Belle,R. (1988) *Eur. J. Biochem.*, **171**, 107–117.
- Norman,C., Runswick,M., Pollock,R. and Treisman,R. (1988) *Cell*, **55**, 989–1003.
- O'Farrell,P.H. (1975) *J. Biol. Chem.*, **250**, 4007–4021.
- Prywes,R. and Roeder,R.G. (1986) *Cell*, **47**, 777–784.
- Prywes,R. and Roeder,R.G. (1987) *Mol. Cell. Biol.*, **1**, 3482–3489.
- Prywes,R., Dutta,A., Cromlish,J.A. and Roeder,R.G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7206–7210.
- Rivera,V.M. and Greenberg,M.E. (1990) *New Biol.*, **2**, 751–758.
- Rozengurt,E. (1986) *Science*, **234**, 161–166.
- Schröter,H., Shaw,P.E. and Nordheim,A. (1987) *Nucleic Acids Res.*, **15**, 10145–10158.
- Schröter,H., Mueller,C.G.H., Meese,K. and Nordheim,A. (1990) *EMBO J.*, **9**, 1123–1130.
- Shaw,P.E., Frash,S. and Nordheim,A. (1989) *EMBO J.*, **8**, 2567–2574.
- Sheng,M., Dougan,S.T., McFadden,G. and Greenberg,M.E. (1988) *Mol. Cell. Biol.*, **8**, 2787–2796.
- Sommercorn,J., Mulligan,J.A., Lozeman,F.J. and Krebs,E.G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8834–8838.
- Stumpo,D., Stewart,T.N., Gilman,M.Z. and Blackshear,P.J. (1988) *J. Biol. Chem.*, **263**, 1611–1616.
- Treisman,R. (1985) *Cell*, **42**, 889–902.
- Treisman,R. (1986) *Cell*, **46**, 567–574.
- Treisman,R. (1987) *EMBO J.*, **6**, 2711–2717.
- Verma,I.M. and Sassone-Corsi,P. (1987) *Cell*, **51**, 513–514.
- Verrier,B., Muller,D., Bravo,R. and Muller,R. (1986) *EMBO J.*, **5**, 913–917.

Received on May 2, 1991; revised on July 4, 1991