The EMBO Journal vol.12 no.10 pp.3903-3911, 1993

Multiple and cooperative phosphorylation events regulate the CREM activator function

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Communicated by P.Sassone-Corsi

Phosphorylation is one of the major mechanisms by which the activity of transcription factors can be regulated. We have investigated the role of phosphorylation in the regulation of the transcription factor CREM. We show that the CREMτ activator is phosphorylated on multiple serine and threonine residues *in vivo*. Stimulation of various signal transduction pathways by forskolin, TPA or Ca^{2+} ionophore leads to enhanced phosphorylation of serine 117, concomitant with an increase in the transactivation potential of CREMτ. We have identified multiple kinases that can also phosphorylate S117 *in vitro*. Moreover, we show that casein kinase I and II cooperatively phosphorylate CREMτ on multiple residues, eliciting enhanced DNA binding. Cooperative phosphorylation is also observed with other kinases. These results show that the activity of CREMτ is regulated by multiple phosphorylation events, suggesting that CREM could be considered as a nuclear effector where signalling pathways may converge and/or cross-talk.

Key words: cyclic AMP/phosphorylation/protein kinase A/signal transduction/transcription factor

Introduction

Transcriptional regulation upon stimulation of the cAMP signalling pathway is mediated by the CREB/ATF family of transcription factors (reviewed in Habener, 1990; Ziff, 1990; Borrelli *et al.*, 1992; Brindle and Montminy, 1992; de Groot and Sassone-Corsi, 1993). These proteins bind as homo- or heterodimers to cAMP-response elements (CREs) present in the promoters of cAMP-regulated genes, thereby modulating the transcription of these genes (Borrelli *et al.*, 1992; Brindle and Montminy, 1992). A member of this family, CREM (CRE modulator) generates both transcriptional activators (CREMτ, τ1 and τ2; Foulkes *et al.*, 1992; Laoide *et al.*, 1993) and repressors (CREMα, β and γ; Foulkes *et al.*, 1991a,b) by alternative splicing. Interestingly, the CREM gene encodes two different bZip DNA binding/dimerization domains, which are alternatively used in the different isoforms (Foulkes *et al.*, 1991a). In addition, we have recently identified another CREM protein, S-CREM, which is generated by alternative translational initiation at an internal AUG codon in CREMτ mRNA (Delmas *et al.*, 1992). Like CREMα, β and γ, S-CREM acts as a repressor of cAMP-induced transcription, probably due to the lack of the KID (kinase inducible domain) region (Delmas *et al.*, 1992). The KID region is common to several other factors of the same family and harbors multiple phosphoacceptor sites for various kinases. Thus, by alternative splicing as well as internal translational initiation, a large number of functionally different proteins are generated from the CREM gene (Foulkes and Sassone-Corsi, 1992).

Over the past 5 years it has become evident that phosphorylation is one of the major mechanisms regulating the activity of transcription factors (reviewed in Hunter and Karin, 1992). Phosphorylation can regulate the activity of transcription factors at multiple levels, including nuclear transport, dimerization, DNA binding and transcriptional activation (Hunter and Karin, 1992). One well characterized example of a transcription factor whose transactivating potential is regulated by phosphorylation, is CREB (CRE binding protein, Hoeffler *et al.*, 1988; Gonzalez *et al.*, 1989). The CREB protein is a weak transcriptional activator in several cell types. However, upon stimulation of the adenylyl cyclase pathway, CREB is phosphorylated on serine 133 by protein kinase A (PKA), which leads to a dramatic increase in its transactivating potential (Gonzalez and Montminy, 1989; Lee *et al.*, 1990). This increase is postulated to result from a phosphorylation-induced conformational change in the CREB protein leading to exposure of glutamine-rich activation domains (Gonzalez *et al.*, 1991). Interestingly, serine 133 in CREB is also phosphorylated upon membrane depolarization in PC12 cells, which again leads to an increase in its transactivating potential (Sheng *et al.*, 1990, 1991; Dash *et al.*, 1991). In this case, however, serine 133 is probably phosphorylated by the calmodulin-dependent kinases I and II (CamKI and II), which are activated upon membrane depolarization in these cells (Sheng *et al.*, 1990, 1991; Dash *et al.*, 1991). Besides serine 133, a number of other serine residues in the KID are phosphorylated *in vivo* (Lee *et al.*, 1990), which can also be phosphorylated *in vitro* by casein kinase II (CK-II). Although these serines are located in a region of CREB that is important for its activity, direct proof that phosphorylation of these residues regulates CREB activity is not yet available.

The activator form of CREM, CREMτ, is highly homologous to CREB in the KID (Foulkes *et al.*, 1992). Since CREMτ is a powerful activator only in the presence of co-transfected PKA (Foulkes *et al.*, 1992; Laoide *et al.*, 1993), we tested whether CREMτ is also phosphorylated by PKA upon activation of the cAMP signalling pathway.

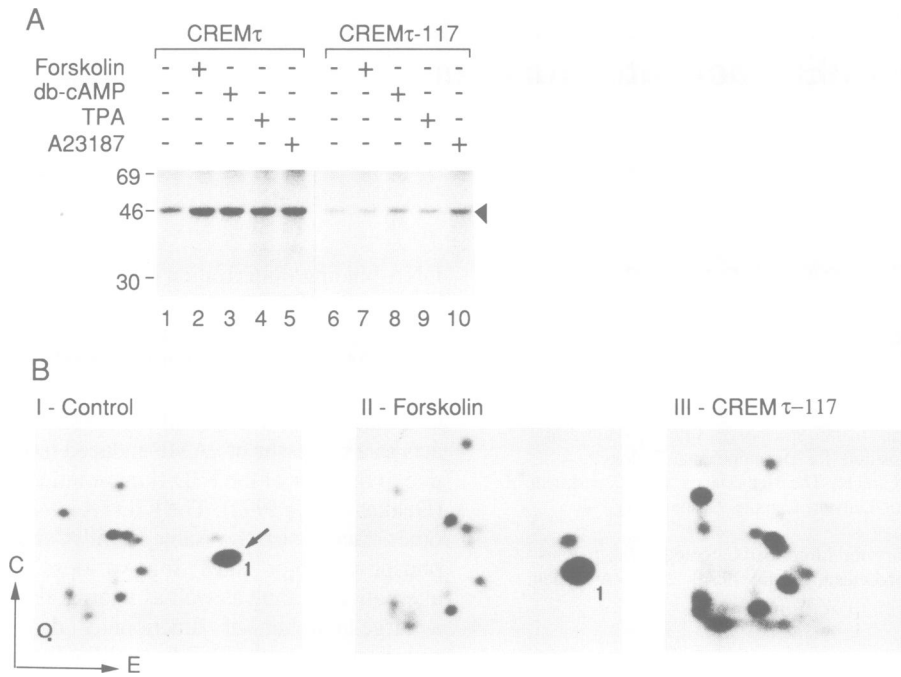

Fig. 1. Constitutive and inducible phosphorylation of CREMτ *in vivo*. (**A**) COS cells were transfected with expression vectors encoding CREMτ or CREMτ-117 (S117 is mutated into alanine). After 48 h, cells were labelled with 1 mCi/ml [^{32}P]orthophosphate for 3 h. Fifteen minutes before harvesting, cells were stimulated with forskolin (20 μM), dibutyryl-cAMP (1 mM), TPA (100 ng/ml), A23187 (2 μM) or carrier alone. CREMτ was then immunoprecipitated and separated on a 10% polyacrylamide gel. (**B**) Tryptic peptide mapping analysis of CREMτ from unstimulated (panel I) or forskolin-treated (panel II) cells. Panel III shows the tryptic peptide map of CREMτ-117 from unstimulated cells. The arrow indicates peptide 1 containing S117. In this and subsequent figures showing tryptic maps, all the spots visible on the TLC plate are presented. O = origin. In all subsequent tryptic maps, the position of the origin is equivalent to O in panel I. C = chromatography; E = electrophoresis.

In this paper we demonstrate that PKA indeed phosphorylates CREMτ on serine 117 (equivalent to serine 133 in CREB) upon activation of the cAMP pathway. In addition, we show that treatment of COS cells with TPA (12-*O*-tetradeconyl-phorbol-13-acetate) or the Ca^{2+} ionophore A23187 also leads to enhanced phosphorylation of serine 117. Moreover, *in vitro* this residue can be phosphorylated by PKA, PKC and CamKII. Significantly, we show that CREMτ can be cooperatively phosphorylated *in vitro* by CK-I and CK-II on residues that were also found to be phosphorylated *in vivo*. CK-I and CK-II appear to be involved in the regulation of the DNA binding capacity of CREMτ. We discuss the importance of these multiple and cooperative phosphorylation events in relation to the function of this transcription factor.

Results

Constitutive and inducible phosphorylation of CREMτ in vivo

Stimulation of the adenylyl cyclase pathway leads to an induction of the transactivation potential of CREMτ (Foulkes *et al.*, 1992; Laoide *et al.*, 1993). Therefore, we investigated whether CREMτ is phosphorylated upon activation of the cAMP-dependent pathway. For this purpose, we transfected COS cells with a CREMτ expression vector, and subsequently labelled the cells with [^{32}P]orthophosphate. Cellular extracts were prepared and immunoprecipitated with a CREM-specific antibody (Delmas *et al.*, 1992). Figure 1A shows that CREMτ is a phosphoprotein even in the absence of activation of the cAMP signalling pathway (lane 1). Treatment of the transfected cells with forskolin or dibutyryl-cAMP leads to a 2-fold enhancement in phosphorylation (lanes 2 and 3). In addition, treatment with the phorbol ester TPA or the Ca^{2+} ionophore A23187 induced a similar rise in phosphorylation (lanes 4 and 5), while the level of CREMτ expression remained constant (not shown). To determine whether serine 117 of CREMτ is a phosphorylation site, we mutated this residue to alanine (CREMτ-117). This mutation leads to a decrease in the basal level phosphorylation of CREMτ (lane 6), as well as complete abolition of the induced phosphorylation (lanes 7–10). These results strongly suggest that serine 117 is a major target for constitutive as well as inducible phosphorylation of CREMτ *in vivo*. We cannot exclude, however, the possibility that serine 117 is not phosphorylated *in vivo*, but that the integrity of serine 117 is necessary for phosphorylation at a distant site. This consideration should be kept in mind with all the mutated residues described throughout this paper. However, since CREMτ-117 still shows a significant level of phosphorylation, it is clear that CREM is also phosphorylated on additional residues. These results were confirmed by performing tryptic peptide mapping analysis on immunoprecipitated CREMτ. Figure 1B shows that tryptic digestion of CREMτ generates a large number of labelled peptides (panel I), of which the major peptide 1 (marked with an arrow), is likely to include serine 117 as is demonstrated by mutating it to alanine (panel III). Tryptic maps of CREMτ from forskolin-stimulated cells show an enhancement of phosphorylation of the peptide including serine 117, while phosphorylation of all the other peptides is very similar (panel II). Similar results were obtained after treatments with dibutyryl-cAMP, TPA and A23187 (not shown).

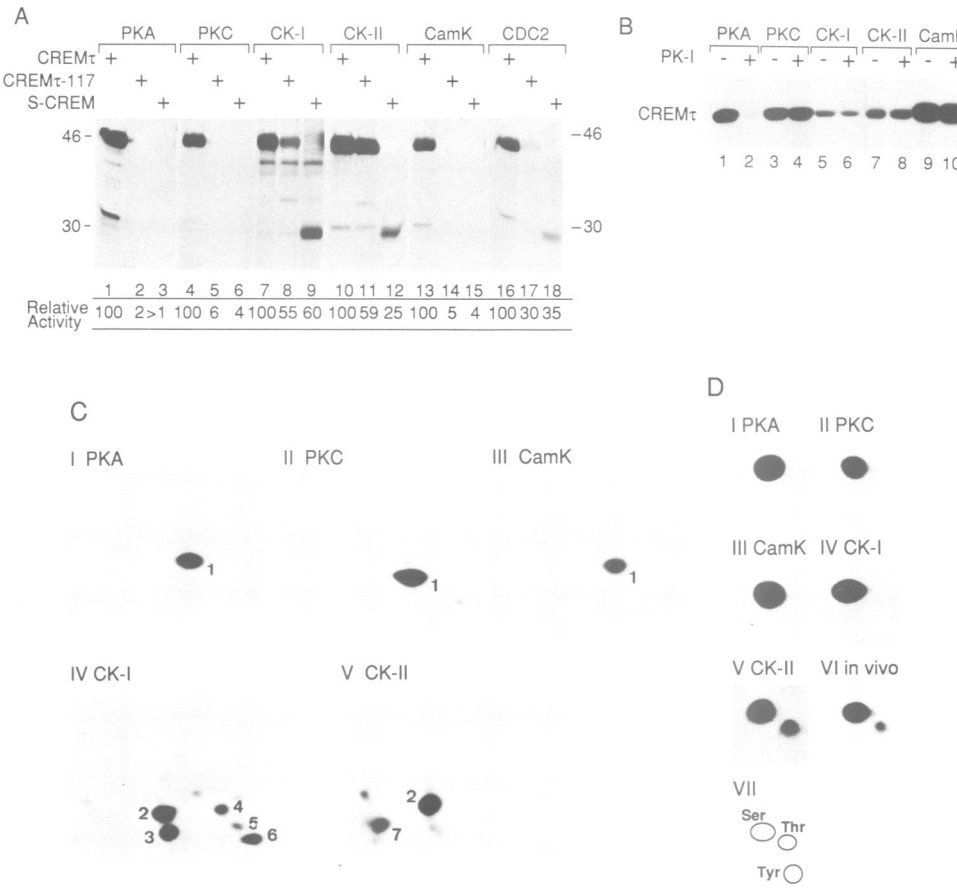

Fig. 2. CREMτ can be phosphorylated by multiple kinases *in vitro*. **(A)** Bacterially expressed CREMτ, CREMτ-117 (45 kDa) or S-CREM (28 kDa) proteins were phosphorylated *in vitro* with PKA, PKC, CK-I, CK-II, CamK or p34^{cdc2}. Samples were loaded on a 10% polyacrylamide gel, and phosphorylated proteins were visualized by autoradiographic exposure of the dried gel. Relative activity was determined by liquid scintillation counting of the excised bands (CREMτ was taken as 100%). While PKA, PKC and CamK phosphorylate only S117, CK-I, CK-II and p34^{cdc2} phosphorylate additional residues. **(B)** CREMτ was phosphorylated with PKA, PKC, CK-I, CK-II or CamK in the absence or presence of the peptide inhibitor of PKA (PK-I; Glass *et al.*, 1989). It is clear that PK-I only inhibits phosphorylation of CREMτ by PKA. **(C)** Tryptic peptide mapping experiments of CREMτ phosphorylated *in vitro* with PKA, PKC, CamK, CK-I or CK-II. While PKA, PKC and CamK only phosphorylate peptide 1 containing S117, CK-I and CK-II phosphorylate multiple distinct peptides (numbered 3–7, see also Figure 3). **(D)** Phospho-amino acid analysis of CREMτ phosphorylated with PKA, PKC, CamK, CK-I or CK-II *in vitro* or CREMτ labelled *in vivo*. PKA, PKC, CamK and CK-I only phosphorylate serine, while CREMτ phosphorylated by CK-II or *in vivo* also contains phospho-threonine.

Multiple kinases phosphorylate CREMτ in vitro

To assess which kinases might be responsible for phosphorylation of CREMτ *in vivo*, we phosphorylated bacterially generated CREMτ, CREMτ-117 and S-CREM proteins *in vitro* using a panel of kinases known to have different recognition sites. The use of S-CREM (Delmas *et al.*, 1992) allows the identification of phosphorylated residues outside the KID area. As shown in Figure 2A, serine 117 of CREMτ is efficiently phosphorylated by PKA (lane 1). Similarly, this residue can also be phosphorylated by PKC (lane 4) and calmodulin-dependent kinase II (CamK; lane 13). This is not the result of contamination of the different kinases by PKA, since a peptide inhibitor of PKA (PK-I; Glass *et al.*, 1989) only abolished phosphorylation of CREMτ by PKA and not that by any other kinase (Figure 2B). As reported elsewhere, CREMτ is also phosphorylated on multiple residues by p34^{cdc2}, S117 being the major phosphorylation site (Figure 2A, lanes 16–18; R.de Groot and P.Sassone-Corsi, submitted). We found that CREMτ is an efficient substrate for CK-I and CK-II (lanes 7–12). These kinases phosphorylate residues distinct from S117, since both CREMτ-117 and S-CREM are phosphorylated by CK-I and CK-II. In contrast, we failed to detect phosphorylation of CREMτ by MAP kinase or kinase FA/GSK-3 (not shown). These results were confirmed and elaborated by tryptic mapping experiments. As shown in Figure 2C, phosphorylation by PKA, PKC or CamKII gives rise to only one labelled peptide which contains S117. By contrast, phosphorylation by CK-I and CK-II gives rise to other labelled peptides. Phospho-amino acid analysis confirms that PKA, PKC and CamKII only phosphorylate a serine residue (Figure 2D). Interestingly, however, while CK-I only phosphorylates serine residues (panel IV), CK-II phosphorylates both serine and threonine residues (panel V). This is likely to be relevant *in vivo*, since CREMτ immunoprecipitated from COS cells contains both phosphoserine and phospho-threonine residues (Figure 2D, panel VI).

Previously it was shown that CREB can be phosphorylated *in vitro* by CK-II on a number of serine residues in the KID (Lee *et al.*, 1990). To determine whether the homologous residues of CREMτ are also phosphorylated by CK-I and CK-II, we mutated these residues to alanine and performed equivalent *in vitro* phosphorylation experiments. As shown in Figure 3A, mutation of S100 inhibits phosphorylation of peptides 3, 5 and 6 by CK-I. This might indicate that peptides

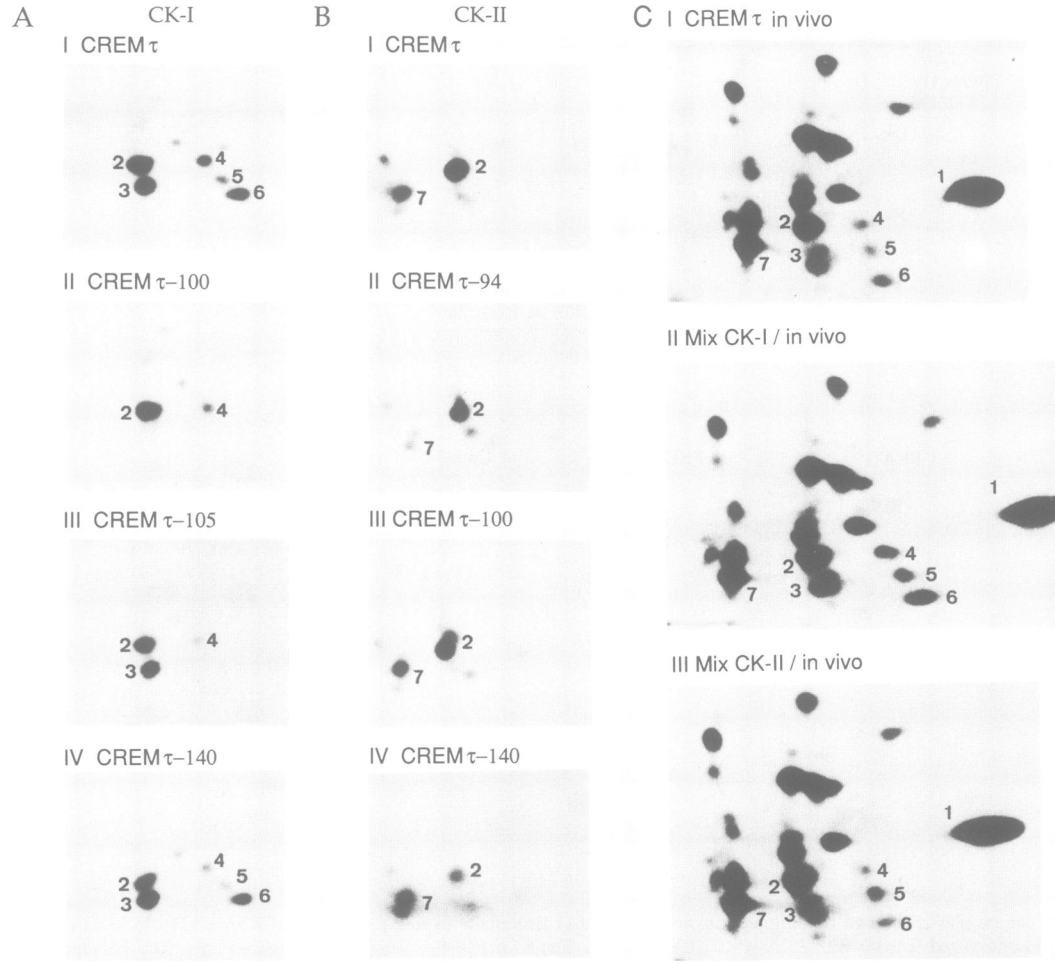

Fig. 3. CK-I and CK-II phosphorylate multiple residues in the KID region of CREMτ. Tryptic peptide mapping experiments of CREMτ phosphorylated *in vitro* with CK-I (**A**) or CK-II (**B**). CK-I phosphorylates at least S100 (panel II), S105 (panel III) and S140 (panel IV), while CK-II phosphorylates at least T94 (panel II) and S140 (panel IV). (**C**) Mixing experiments of peptide maps obtained from CREMτ labelled *in vivo* (COS cells, panel I) with CREMτ labelled *in vitro* by CK-I (panel II) and CK-II (panel III). It is clear that peptides 2–7, which are phosphorylated by CK-I and CK-II (see panels A and B), are also observed in the tryptic map from COS cells. However, to detect peptides 3–6, the maps needed to be overexposed compared with the *in vivo* maps of Figure 1.

3, 5 and 6 are partially digested fragments containing only phosphorylated S100, or that these peptides contain multiple phosphorylated residues that are all dependent on S100. Although at present we do not have an explanation for the disappearance of three peptides, this suggests that S100 is phosphorylated by CK-I. Similarly, S105 is likely to be phosphorylated by CK-I, since mutation of S105 blocks phosphorylation of peptides 5 and 6. Finally, mutation of S140 decreases the phosphorylation of peptide 2 by both CK-I as well as CK-II by ~50%, indicating that S140 is likely to be phosphorylated by both kinases. This result also suggests that peptide 2 in fact is a doublet of two co-migrating peptides that are both phosphorylated by CK-I as well as CK-II. By contrast, CK-II is likely to phosphorylate T94, since mutation of this residue inhibits phosphorylation of peptide 7. This result is in agreement with the phospho-amino acid analysis of Figure 2D, showing that CK-II phosphorylates CREM on both serine and threonine residues.

To determine whether the residues phosphorylated by CK-I and CK-II *in vitro* were also phosphorylated *in vivo*, we performed mixing experiments using tryptic digestions of *in vitro* and *in vivo* labelled CREMτ protein. As shown in Figure 3C, all the tryptic peptides observed in the *in vitro*

Table I. Summary of amino acids phosphorylated *in vitro* and *in vivo*

Kinase	Residue	Peptide	*In vivo*
PKA	S117	1	+++
PKC	S117	1	+++
CamK	S117	1	+++
CK-I	S100	3, 5, 6	+
	S105	5, 6	+
	S140	2	++
	?	2	++
CK-II	T94	7	++
	S140	2	++
	?	2	++
?	S126	8, 9	++
?	S127	8, 9	++
FA/GSK-3	S113	10	–
p34^{cdc2}	S117	1	+++
	S140	2	++
	T?	2	++
	S268	11, 12	++
	S274	13	+

+ weak, ++ strong, +++ very strong

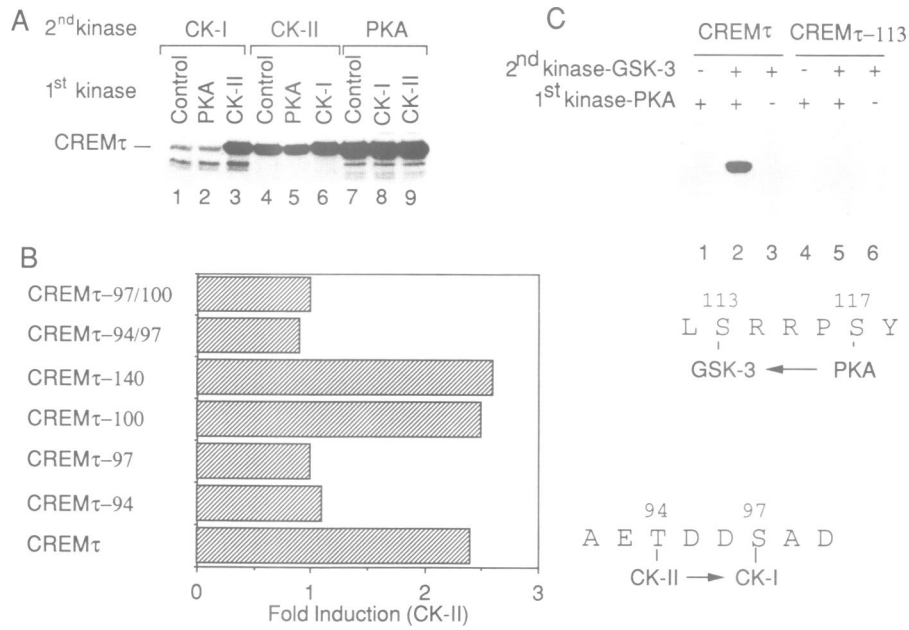

Fig. 4. Cooperative phosphorylation of CREMτ *in vitro*. **(A)** Bacterially produced CREMτ was pre-phosphorylated (first kinase) for 30 min with CK-I, CK-II, PKA or buffer alone (control) in the absence of [γ-^{32}P]ATP. After heating for 5 min at 80°C and cooling down to 30°C, the second kinase and [γ-^{32}P]ATP were added for another 30 min incubation. Pre-phosphorylation with CK-II clearly enhances phosphorylation by CK-I. **(B)** The effect of pre-phosphorylation with CK-II on phosphorylation by CK-I was tested using CREMτ and different mutant CREMτ proteins (numbers represent S or T residues mutated to alanine). Fold induction represents enhanced phosphorylation by CK-I after pre-phosphorylation with CK-II. The right panel shows that when T94 is phosphorylated by CK-II, S97 becomes a good substrate for CK-I. **(C)** CREMτ or CREMτ-113 proteins were treated with PKA or buffer in the presence of unlabelled ATP. After heating for 5 min at 80°C and cooling down to 30°C, kinase FA/GSK-3 and [γ-^{32}P]ATP were added for another 30 min incubation. Kinase FA/GSK-3 phosphorylates S113 only when S117 is pre-phosphorylated by PKA.

maps of CK-I and CK-II co-migrate with peptides labelled *in vivo*. However, to detect the phosphorylation of peptides 3−6 *in vivo*, the map needed to be overexposed, indicating that these sites are not strongly phosphorylated *in vivo* (Table I). These results are in agreement with the phosphorylation of CREB on homologous residues in COS cells (Lee *et al.*, 1990).

Cooperative phosphorylation by various kinases

It is known that pre-phosphorylation by a specific kinase can, in some cases, enhance the subsequent activity of another kinase on a distinct phosphoacceptor site. Well documented examples are glycogen synthase (Flotow and Roach, 1989) and the modulator subunit of protein phosphatase 1 (DePaoli-Roach, 1984; Agostinis *et al.*, 1992). As was reported for glycogen synthase, pre-phosphorylation of CREMτ enhances phosphorylation by CK-I. When CREMτ was pre-phosphorylated with CK-II, CK-I phosphorylation was significantly stimulated, while pre-phosphorylation by PKA had no such effect (Figure 4A, lanes 1−3). Interestingly, the converse experiment, where CREM was pre-phosphorylated by CK-I, did not lead to enhanced phosphorylation by CK-II (lanes 4−6). By employing the different mutated CREMτ proteins, we were able to demonstrate that the cooperation was dependent on sites T94 and S97 (Figure 4B). When CK-II phosphorylates T94 (see Figure 3B), S97 becomes a good substrate for CK-I, which fits well with the described S^{P}/T^{P}XXS*/T* consensus sequence for CK-I (Pearson and Kemp, 1991).

A similar cooperation was found between PKA and kinase FA/GSK-3 (Figure 4C). Kinase FA/GSK-3 by itself does not phosphorylate CREMτ (lane 3), but when S117 is pre-phosphorylated by PKA, CREMτ is a good substrate for kinase FA/GSK-3 (lane 2). Serine 113 is the target site for kinase FA/GSK-3, since mutation of S113 to alanine completely abolishes phosphorylation by kinase FA/GSK-3 (lane 5). This might be relevant *in vivo*, since S117 is phosphorylated in the absence of mitogenic signalling (Figure 1B). A comparable situation was previously found for the G subunit of protein phosphatase 1 (PP1G), where pre-phosphorylation of one serine residue is necessary for kinase FA/GSK-3 to phosphorylate another serine residue four amino acids closer to the N-terminus (Dent *et al.*, 1989).

The *in vivo* relevance of CREM phosphorylation by FA/GSK-3 is unclear, since we failed to identify phosphorylation of S113 in COS cells, indicating that kinase FA/GSK-3 does not phosphorylate CREMτ in these cells (Table I). However, when a kinase FA/GSK-3 expression vector was co-transfected with CREMτ, peptide 10 containing S113 was phosphorylated (arrow in Figure 5A, panel II), indicating that the lack of phosphorylation of S113 in COS cells may be due to the absence of active kinase FA/GSK-3 in these cells.

Previously it was shown that the sequence DLSSD (aa 125−129) in the CREB KID is important for transactivation (Lee *et al.*, 1990). Since this sequence is highly conserved in CREMτ (124-ELSSD-128), we tested whether S126 and S127 might be phosphorylated *in vivo*. Mutation of either S126 or S127 to alanine leads to a strong decrease in the phosphorylation of peptides 8 and 9 (Figure 5B). This result suggests that peptides 8 and 9 are partially digested fragments containing phosphorylated S126 and S127, and that the phosphorylation of either residue is dependent on the phosphorylation of the other residue. As yet, we have failed to identify the kinase that phosphorylates these residues *in*

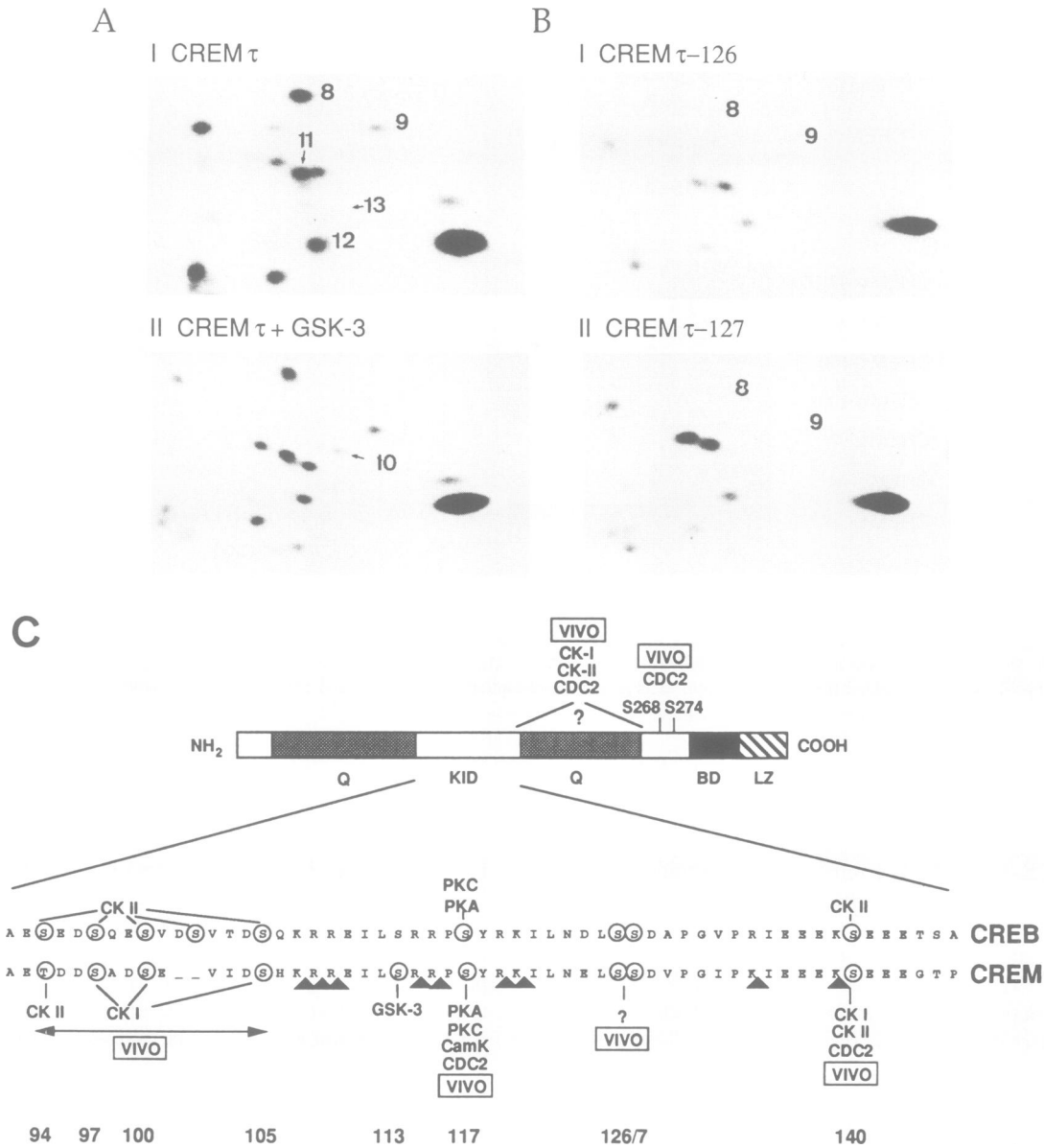

Fig. 5. Phosphorylation of S113, S126 and S127 *in vivo*. (**A**) Tryptic peptide mapping experiments of CREMτ immunoprecipitated from COS cells in the absence (panel I) or presence (panel II) of co-transfected kinase FA/GSK-3 expression vector. Kinase FA/GSK-3 expression leads to the appearance of a novel peptide containing S113 (peptide 10 indicated by an arrow). (**B**) Tryptic peptide mapping experiments of CREMτ-126 (panel I) or CREMτ-127 (panel II) immunoprecipitated from COS cells. Mutation of either S126 or S127 to alanine leads to the disappearance of peptides 8 and 9. (**C**) Schematic representation of the structure of CREMτ and the localization of the phosphorylated residues. The detailed structure of the kinase inducible domain (KID) and a comparison with the CREB KID are shown. Numbers indicate serine or threonine residues (circled) in CREMτ phosphorylated by the indicated kinases. VIVO indicates amino acids that are phosphorylated in COS cells. Triangles indicate trypsin cleavage sites. Q, glutamine-rich activation domain; BD, basic domain; LZ, leucine zipper.

vivo, although CamKII does phosphorylate S126 and S127 *in vitro* when high enzyme concentrations are used (not shown). As has been demonstrated elsewhere, peptides 11 and 12 (which include S268) and peptide 13 (which includes S274) can be efficiently phosphorylated by p34^{cdc2} *in vitro* (R.de Groot *et al.*, submitted). A summary of all the residues identified as being targets for phosphorylation by various kinases is presented in Figure 5C.

Phosphorylation modulates DNA binding and transactivation by CREMτ

CK-II has recently been implicated in the regulation of DNA binding by sequence-specific transcription factors. Phosphorylation of the serum response factor (SRF; Treisman, 1986) by CK-II leads to enhanced DNA binding (Janknecht *et al.*, 1992). By contrast, CK-II negatively regulates DNA binding by the c-Jun transcription factor (Lin *et al.*, 1992). To determine whether CK-I and CK-II modulate DNA binding by CREMτ, we tested *in vitro* phosphorylated CREMτ protein in a gel shift assay. As shown in Figure 6, phosphorylation of CREMτ by either CK-I (panel A) or CK-II (panel B) leads to a 3-fold enhancement in binding to a CRE. In contrast, phosphorylation by PKA, PKC or CamKII did not affect DNA binding by CREMτ (not shown). Similar results were obtained using CREB (not shown). The modulation of DNA binding by

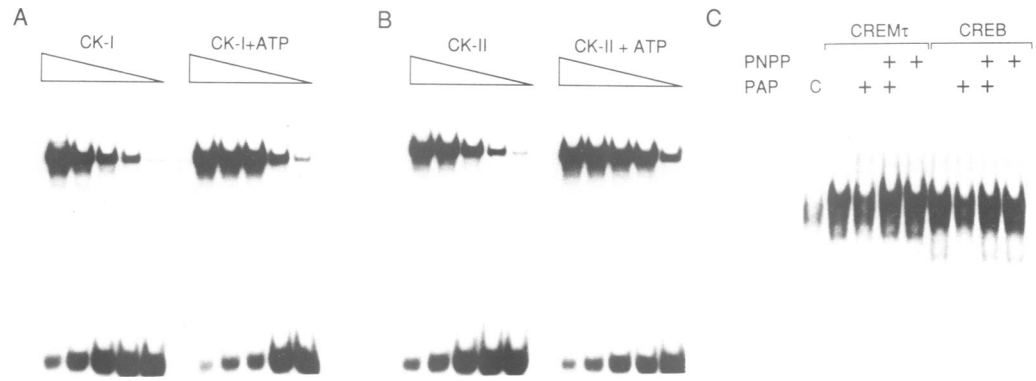

Fig. 6. CK-I and CK-II enhance DNA binding by CREMτ. (**A** and **B**) Bacterially produced CREMτ was treated with CK-I (A) or CK-II (B) in the absence or presence of ATP, after which serially diluted samples (from 0.3 μg to 4 ng) were tested in a gel mobility shift assay using a ^{32}P-labelled CRE oligonucleotide. Phosphorylation enhances DNA binding by CREMτ. (**C**) COS cells were transfected with CREMτ or CREB expression vectors, after which whole cell extracts were prepared. Extracts were treated with potato acid phosphatase (PAP) for 30 min at 30°C. To block PAP activity, 10 mM *p*-nitrophenyl phosphate (PNPP) was included in some reactions. Samples were then tested for DNA binding activity in a gel mobility shift assay. De-phosphorylation by PAP reproducibly results in a 50% decrease in DNA binding activity of CREMτ and CREB (established by liquid scintillation counting), while this decrease is blocked by treatment with PNPP.

phosphorylation might have potential relevance *in vivo*, since DNA binding of CREMτ or CREB produced in transfected COS cells is decreased upon treatment with potato acid phosphatase (Figure 6C). Thus, these results suggest that CK-I and CK-II are positive regulators of DNA binding by CREMτ and CREB *in vivo*.

Phosphorylation is also reported to influence dimerization of transcription factors (Hunter and Karin, 1992). It has been suggested that phosphorylation by PKA enhances CREB dimer formation (Yamamoto *et al.*, 1988). However, using bacterially produced CREMτ and CREB, we were unable to detect an effect of any of the kinases tested here upon either homo- or heterodimerization (not shown).

Stimulation of different signalling pathways leads to enhanced phosphorylation of S117 (Figure 1A). To determine the effect of this increase on transactivation by CREMτ, co-transfections were performed in COS cells. As shown in Figure 7, a GAL4−CREMτ fusion protein (G4CREMτ) weakly activates transcription of a G4−CAT reporter construct. When the cells are stimulated with forskolin, the activity of G4CREMτ is increased 10-fold. This increase is dependent on S117, since mutating this residue to alanine leads to a strong decrease in basal level activity of G4CREMτ and to a complete block of the forskolin-dependent induction. Stimulation with TPA or A23187 leads to a modest increase in G4CREMτ activity. Again, this increase is dependent on S117 (Figure 7). These results show that induced phosphorylation of CREMτ on S117 in response to forskolin, TPA and A23187 leads to enhanced transactivation by CREMτ.

We have also tested the CREM proteins in co-transfection assays in which the residues that are phosphorylated by CK-I, CK-II or FA/GSK-3 were mutated. However, these mutations did not significantly alter PKA-dependent activation of transcription by CREMτ (data not shown). It might well be that in order to modulate significantly the transactivation by CREM, multiple residues need to be mutated.

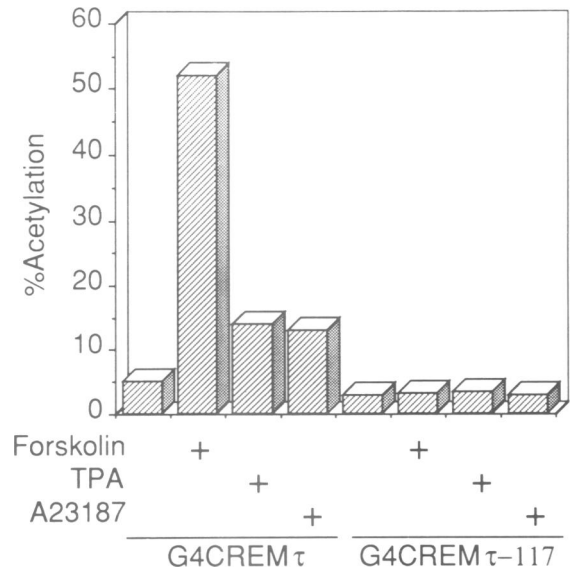

Fig. 7. Induced phosphorylation of S117 leads to enhanced transactivation by CREMτ. COS cells were transiently transfected with expression vectors encoding fusion proteins of GAL4 and wild type or S117-A mutant CREMτ (G4CREMτ and G4CREMτ-117) and a reporter plasmid containing the CAT gene and a GAL4 binding site. After 24 h, cells were treated with forskolin (10 μM), TPA (100 ng/ml) or A23187 (2 μM) for 8 h before harvesting and determination of the CAT activity. Stimulation with forskolin, TPA or A23187 enhances transactivation by G4CREMτ, but not by G4CREMτ-117. The data reported in this figure are representative; variation in the results was never higher than 10%.

Discussion

Phosphorylation is one of the major mechanisms by which the activity of transcription factors can be regulated (Hunter and Karin, 1992). In this paper we demonstrate that the transcription factor CREMτ is phosphorylated on multiple residues *in vivo*. Different intracellular signal transduction pathways converge to enhance phosphorylation of S117 and thus to an increased transactivation by CREMτ. In addition we have shown that while *in vitro* phosphorylation of S117 by PKA, PKC and CamKII does not modulate DNA binding by CREMτ, phosphorylation by CK-I and CK-II is implicated in the regulation of the DNA binding capacity of CREMτ.

Other members of the CREB/ATF family are activated through the adenylyl cyclase pathway. CREB is rapidly

phosphorylated by PKA on S133 upon cAMP pathway activation, leading to a strong increase in its *trans*-regulatory function (Gonzalez and Montminy, 1989). Similarly, ATF-1 (Hai *et al.*, 1989) is activated when co-transfected with the catalytic subunit of PKA, although phosphorylation of ATF-1 by PKA *in vivo* remains to be demonstrated (Flint and Jones, 1991; Rehfuss *et al.*, 1991). Moreover, as we demonstrate here for CREMτ, both CREB and ATF-1 are activated by Ca^{2+}-dependent signalling (Sheng *et al.*, 1990, 1991; Dash *et al.*, 1991), although direct phosphorylation of ATF-1 by CamKII remains to be demonstrated. In this paper we show that phosphorylation of S117 of CREMτ is enhanced upon stimulation by cAMP, Ca^{2+} ionophore and TPA. Therefore, proteins of this class should be considered as nuclear targets for multiple signal transduction pathways. The convergence of these pathways is likely to be involved in the transcriptional regulation of genes that are induced via multiple signalling cascades. At the moment, we can only speculate about the function of the apparent redundancy in the CREB/ATF family. It is possible that some differences in the DNA binding specificity exist between CREB, ATF-1 and CREMτ, and that these three proteins might perform their overlapping function in different cell types or at different times during development. Indeed, we have previously shown that while CREB seems to be ubiquitously expressed, CREMτ expression is highly regulated by cell-specific mechanisms (Foulkes *et al.*, 1991a, 1992).

It has been reported previously that CREB can be phosphorylated on at least three residues by PKC *in vitro*. In addition, it has been shown that PKC enhances DNA binding of CREB through stimulation of dimer formation (Yamamoto *et al.*, 1988). Using bacterially produced CREB and CREMτ proteins, we detected only a single PKC phosphorylation site (S117, see Figure 2A) and also we did not observe changes in dimerization or DNA binding upon phosphorylation of CREMτ or CREB by PKC. This discrepancy might be related to the different source of CREB protein, since Yamamoto *et al.* (1988) have used CREB isolated from rat brain, which could be phosphorylated at additional sites. However, we could not modify the PKC-induced phosphorylation pattern by pre-phosphorylation with other kinases. Thus at present, we do not have a complete explanation for the differences between our data and the results obtained by Yamamoto *et al.* (1988). Whether PKC phosphorylates CREMτ *in vivo* remains to be demonstrated, although the enhanced phosphorylation of S117 *in vivo* upon TPA treatment suggests that PKC might directly phosphorylate CREMτ. Alternatively, PKC might activate another kinase, which in turn phosphorylates S117.

Whether phosphorylation by PKA influences DNA binding of CREB and CREMτ remains a controversial point. In a study by Nichols *et al.* (1992), a 2- to 3-fold increase in DNA binding by CREB was observed upon phosphorylation by PKA. In contrast, others have failed to obtain similar results (Yamamoto *et al.*, 1988). In this paper we have shown that neither PKA, nor other kinases that phosphorylate S117, modulate DNA binding or dimerization by CREMτ or CREB. It is more likely that phosphorylation of S117 might lead to a conformational change in CREMτ that exposes the glutamine-rich activation domains, as was previously demonstrated for phosphorylation of CREB by PKA (Gonzalez *et al.*, 1991). Our data on CREM indicate that induction of DNA binding by phosphorylation is obtained only through the action of CK-I and CK-II on phosphoacceptor sites other than S117.

In this paper we have shown that CREMτ cannot be phosphorylated by kinase FA/GSK-3 *in vitro*. However, when S117 was pre-phosphorylated by PKA (or another kinase), S113 becomes an efficient substrate for kinase FA/GSK-3 *in vitro*. Since we have shown that S117 is phosphorylated *in vivo* (Figure 1), it is possible that kinase FA/GSK-3 regulates CREMτ activity *in vivo*. Indeed, when we co-transfected a kinase FA/GSK-3 expression vector into COS cells, S113 was phosphorylated. The absence of phosphorylation of S113 in COS cells is therefore likely to be the result of a reduced FA/GSK-3 kinase activity in these cells. However, the function of phosphorylation by FA/GSK-3 kinase remains unclear, since we failed to detect an effect upon CREMτ DNA binding or transactivation (not shown).

We also show that CREMτ can be phosphorylated *in vitro* by CK-II in the KID region on residues that are also phosphorylated *in vivo*. Moreover, we have shown that CK-I also phosphorylates a number of these residues, and that pre-phosphorylation by CK-II enhances phosphorylation by CK-I. Interestingly, we found that phosphorylation by CK-I and CK-II enhances DNA binding by CREMτ. This might be relevant *in vivo*, since dephosphorylation of CREMτ isolated from COS cells leads to a decrease in DNA binding. CK-II was previously implicated in the regulation of DNA binding by SRF, where phosphorylation by CK-II enhances DNA binding by SRF through increasing the rate of SRF binding site exchange (Janknecht *et al.*, 1992). In contrast, CK-II seems to be involved in the negative regulation of DNA binding by c-Jun (Lin *et al.*, 1992). Elucidation of the mechanism whereby CK-I and CK-II enhance DNA binding by CREMτ awaits further characterization.

In conclusion, the activity of CREMτ is regulated by multiple constitutive as well as inducible phosphorylation events. In addition, cooperative phosphorylation is possible. Therefore, besides being a component in the adenylyl cyclase pathway, CREM is likely to act as a nuclear effector where other distinct signalling pathways may converge and/or cross-talk. Analysis of the *in vivo* function of CREMτ phosphorylation will contribute to a better understanding of the role of these events in gene expression and physiology.

Materials and methods

Cells and plasmids

COS and Swiss 3T3 cells were cultured in DMEM containing 5% and 10% fetal calf serum, respectively. G4CREMτ, G4CREMτ-117 and G4–CAT have been described elsewhere (Laoide *et al.*, 1993). The kinase FA/GSK-3 expression vector was generously given by J.Woodgett. The PKA expression vector was generously given by S.G.McKnight.

For *in vitro* mutagenesis experiments, the CREMτ cDNA was cloned in M13 mp18. Mutagenesis was performed using the Bio-Rad mutagenesis kit. The following oligonucleotides were used (mutations are underlined). Threonine 94: T GCA GAG GCA GAT GAT; serine 97: A GAT GAT GCT GCA GAC; serine 100: T GCA GAC GCA GAA GTA; serine 105: A ATT GAT GCG CAT AAA; serine 113: A ATT CTT GCA CGA AGA; serine 117: AGA CCC GCA TAT AGA A; serine 126: T GAA CTT GCC TCT TGAT; serine 127: A CTT TCC GCT GAT GTG; serine 140: A GAA AAA GCA GAG GAA. After verification of the mutations by sequencing, the mutant cDNAs were cloned in the mammalian expression vector pSG5 (Green *et al.*, 1988) for transfection in COS cells and in the bacterial expression vector pET11D (Studier *et al.*, 1990) for the production of recombinant protein in *Escherichia coli*.

Transient transfection

Transient transfections were performed in COS and Swiss 3T3 cells by the calcium phosphate co-precipitation technique. 24 h after transfection, the cells were treated with forskolin (20 μM), TPA (100 ng/ml), A23187 (2 μM) or carrier for 8 h, after which the cells were harvested and assayed for CAT activity .

In vitro and in vivo phosphorylations

Expression and purification of CREM proteins from bacteria have been described previously (Delmas *et al.*, 1992; R.de Groot and P.Sassone-Corsi, submitted). 1 μg of protein was incubated in a 15 μl volume with 2–10 picomolar units of PKA (Sigma), PKC (generously given by S.Stabel), CamKII (generously given by H.Schulman), CK-I, CK-II or kinase FA/GSK-3. CK-I and CK-II were prepared from porcine spleen essentially as in Meggio *et al.* (1981), with an extra final ion exchange chromatography purification step (Mono S in the case of CK-I and Mono Q in the case of CK-II). Kinase FA/GSK-3 was purified from bovine brain as described previously (Vandenheede *et al.*, 1980), with an additional Mono S chromatography step to separate the α from the β isoform (Van Lint *et al.*, in preparation). Standard reactions contained 10 mM Tris pH 7.4, 20 mM $MgCl_2$, 50 μM ATP and 10 mCi/ml [γ-^{32}P]ATP. For CamKII, 0.5 mM $CaCl_2$ and 20 μg/ml calmodulin were included (Schulman *et al.*, 1985). After incubation for 10–30 min at 30°C, reactions were stopped by the addition of Laemmli sample buffer and analyzed on 10% polyacrylamide gels. In some reactions, peptide inhibitor of PKA (PK-I, Glass *et al.*, 1989; 10 μM, Sigma) was included.

For cooperative phosphorylation assays, CREMτ was pre-phosphorylated by the first kinase with unlabelled ATP. After 10–30 min, the kinase was inactivated by heating at 80°C for 5 min, followed by the addition of the second kinase and [γ-^{32}P]ATP. Heat treatment is not likely to change the native structure of CREM, since the protein was boiled during isolation (Delmas *et al.*, 1992). Controls without the second kinase were performed to ensure that the first kinase was completely inactivated.

For *in vivo* phosphorylation, COS cells were transfected with CREMτ expression vectors by the standard calcium phosphate procedure 2 h after splitting. Cells were labelled with [^{35}S]methionine (500 μCi/ml) or [^{32}P]orthophosphate (1 mCi/ml) for 3 h, followed by immunoprecipitation of CREMτ as described previously (Delmas *et al.*, 1992). Cells were treated with forskolin (20 μM), TPA (100 ng/ml), A23187 (2 μM), or carrier for 15 min before harvesting. Proteins were separated on a 10% polyacrylamide gel.

Tryptic peptide mapping and phospho-amino acid analysis

Tryptic peptide mapping and phospho-amino acid analyses were performed essentially as described by Boyle *et al.* (1991). In short, labelled protein was cut out from dried gels, eluted in ammonium bicarbonate, precipitated with TCA and digested with trypsin. After digestion, samples were washed and lyophilized three times. Labelled peptides were separated by electrophoresis in pH 1.9 buffer in the first dimension, followed by chromatography in the second dimension.

For phospho-amino acid analysis, TCA precipitated protein was hydrolyzed in 6 M HCl for 1 h at 110°C. Phospho-amino acids were separated by two-dimensional electrophoresis (pH 1.9 followed by pH 3.5), and were identified by co-migration of unlabelled phospho-amino acid standards (Boyle *et al.*, 1991).

Gel mobility shift assays

Gel mobility shift assays using bacterially produced CREMτ and CREB proteins and the CRE from the somatostatin promoter were performed as described previously (Delmas *et al.*, 1992). The effect of phosphorylation on DNA binding was tested by treating CREMτ protein with kinase in the presence or absence of ATP. Serial dilutions were then tested for DNA binding. For dimerization assays, proteins were heated at 90°C for 5 min followed by cooling down to room temperature over a period of 30 min.

The effect of dephosphorylation on DNA binding by CREMτ was tested by isolating whole cell extracts from transfected COS cells as described previously. 10 μg of extract was then treated with 1 μg of potato acid phosphatase (Boehringer) for 30 min at 30°C in the absence or presence of 10 mM *p*-nitrophenyl-phosphate (Boehringer). Samples were then tested in a gel shift assay as described by Laoide *et al.* (1993).

Acknowledgements

We would like to thank S.Stabel, S.G.McKnight, J.Woodgett and H.Schulman for various gifts of material. We also thank V.Delmas, N.S.Foulkes, B.M.Laoide, D.Masquilier and C.A.Molina for discussions and F.Schlotter for help. R.d.G. was supported by a Dutch Cancer Society fellowship. J.R.V. and J.G. were supported by the 'Geconcentreerde Onderzoeksacties van het Ministerie van de Vlaamse Gemeenschap'. This work was supported by grants from the CNRS, INSERM, FRM, CHUR, Association pour la Recherche contre le Cancer and by Rhône-Poulenc Rorer to P.S.-C.

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Received on January 14, 1993; revised on June 1, 1993