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Multisite phosphorylation of the cAMP response element-binding protein (CREB) by a diversity of protein kinases

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1. ABSTRACT

The prevailing view of stimulus-induced activation of the transcription factor cAMP response element-binding protein (CREB) presumes phosphorylation at serine-133. Although, phosphorylation of this residue seems to be necessary, it is not sufficient to trigger CREB-driven transcription, indicating that other phosphoserine-133-independent mechanisms are required for full activation of CREB. One of these additional regulatory mechanisms influencing the transcriptional state of CREB may involve multiple phosphorylation events on other phosphoacceptor sites in the protein. This review focuses on the phosphorylation modifications of CREB by distinct protein kinases and discusses the possible implications for the function of CREB.

2. INTRODUCTION

Post-translational modification is an important mechanism that contributes to the regulation of proteins, including transcription factors. Phosphorylation is the best-studied and most abundant modification of proteins and is controlled by the antagonistic action of protein kinases and protein phosphatases. The human genome project has led to the identification of approximately 520 different protein kinases and ~110 protein phosphatases (1-3). It is estimated that about 30% of all proteins in the cell are transiently phosphorylated at one or multiple amino acid residues (4,5). Among the phosphoproteins are transcription factors and their coregulators. It is assumed that most of the nearly 1000 transcription factors in the genome are regulated in some fashion by phosphorylation (6). Phosphorylation can affect the transcriptional activity of a transcription factor

and its coregulators by: (i) controlling their nuclear localization or the subcellular localization of the protein kinases and protein phosphatases that catalyze their phosphorylation and dephosphorylation; (ii) modulating protein-protein interactions; (iii) targeting them for proteolytic degradation; (iv) regulating the DNA binding of the transcription factor; (v) changing chromatin structure (reviewed in 7). Although phosphorylation of a transcription factor on a single phosphoacceptor site may be sufficient for binary on-off regulation, multiple phosphorylations may cause gradually increment of the activity of the protein (reviewed in 6).

The transcription factor cyclic AMP (cAMP) response element-binding protein (CREB) is a prototype of a protein whose activity is highly affected by phosphorylation. This 43 kDa protein was originally isolated as a transcription factor that binds to the palindromic sequence TGACGTCA in the somatostatin promoter. The 8 base-pair motif was identified in numerous other cAMP-inducible genes and was therefore referred to as the cAMP response element (CRE). In its unphosphorylation state, CREB possessed low transcriptional activity. The cAMP-dependent protein kinase or protein kinase A (PKA) was shown to phosphorylate CREB exclusively at serine-133 in vivo, a modification that strongly enhances the transcriptional activity of CREB. Mutation of this residue into alanine resulted in a transcriptionally inactive CREB. Acidic substitutions (serine into aspartic acid or glutamic acid) to mimic phosphorylation could not restore the transcriptional potentials of CREB. Subsequent studies showed that this phosphorylation event enables CREB to recruit the coactivator CBP (reviewed in 8 and 9). The molecular mechanism for phosphoserine-133-dependent interaction between CREB and CBP has been resolved. The domain of CREB that interacts with CBP is known as the kinase-inducible domain or KID, which encompasses serine-133, while KIX constitutes the region of CBP that binds KID. The KIX domain contains helical structures, while phosphorylated KID undergoes a transition from a coiled conformation to a helix folding upon binding to KIX, forming two alpha helices that interact with the alpha helices of KIX. The phosphate group of the critical phosphoserine-133 residue forms a hydrogen bond to the side chain of Tyr-658 of KIX (10,11). CBP possesses histone acetyltransferase activity, and has been suggested to mediate target gene activation, in part, by acetylating and disrupting promoter-bound nucleosomes. Moreover, it can link CREB to the basal transcription machinery as CBP was found to indirectly contact RNA polymerase II by the bridging protein RNA helicase A (8).

Serine at position 133 constitutes the major phosphorylation site, but other residues can act as phosphoacceptor sites and to date, more than 20 different protein kinases have been shown to phosphorylate CREB (Figure 1 and Table 1). Here we review the known CREB kinases and discuss the biological relevance of CREB phosphorylation events at these different sites.

3. CRITERIA THAT CLASSIFY A PROTEIN KINASE AS A *BONA FIDE* CREB KINASE

Although several protein kinases have been shown to be involved in mediating stimulus-induced phosphorylation of CREB, evidence for direct *in vivo* interaction between the kinase and its substrate CREB resulting in phosphorylation is often lacking. Phosphorylation of purified CREB by a particular protein kinase in an *in vitro* kinase or an *in vitro* in-gel kinase assay indicates a direct role for the protein as a CREB kinase. Phosphorylation of endogenous CREB in cells by transient ectopic expression of an activated variant of the kinase and inhibition of CREB phosphorylation by specific pharmaceutical inhibitors, inhibiting peptides, kinase deficient mutants, siRNA or knock-out models targeting the kinase further underscores a role for the kinase as a CREB kinase. Interaction studies by e.g. yeast two-hybrid assay or co-immunoprecipitation provide addition proof that the kinase is a genuine CREB kinase. Finally, the presence of a consensus phosphorylation motif for a specific kinase in CREB and the subcellular colocalization and cellular coexpression of the kinase and CREB are indicative for a putative function of the enzyme as a CREB kinase.

4. CREB KINASES

4.1. Ataxia-telangiectasia mutated (ATM)

The *ATM* gene encodes a protein kinase belonging to the phosphoinositide 3-kinase-related gene superfamily. ATM activating stimuli like irradiation and H_2O_2 induced a time- and dose-dependent phosphorylation of CREB, but on (a) site(s) distinct from serine-133 in K562 cells. CREB has 6 putative ATM and ATR (ATM-Rad3-related kinase) phosphoacceptor sites (i.e. SQ or TQ motifs): T-100, S-111, S-121, T-186, T-215, T-256. The first three potential phosphoacceptor sites are part of the KID, while T-186 and T-215 are part of the Q2 domain. T-256 does not reside in any of the transactivation domains of CREB, but is located in the DNA-binding/dimerization basic leucine zipper (bZIP) domain in the C-terminal part of CREB. Mutation analyses studies showed that CREB is phosphorylated on T-100, S-111 and S-121, but not T-186, T215 and T-256, by ATM-activating stimuli in cells with functional ATM. Phosphorylation of S-111 is required for phosphorylation of S-121 as the S111A mutation completely blocked H_2O_2-induced phosphorylation of S-121. GST-KIX pulldown studies revealed that irradiation prevented the interaction between wild-type CREB with the KIX domain of CBP, but had no effect on the binding of the triple T100A/S111A/S121A mutant. Thus ATM-induced phosphorylation of T-100, S-111, S-121 antagonizes CREB-KIX interaction, while phosphorylation of Ser-133 counteracted this inhibitory effect (12). In a more recent study by the same group, commercially available phosphoserine-121 specific antibodies were applied. ATM and ATR phosphorylated CREB at serine-121 *in vitro*, however, the ATM but not the ATR activation status correlated with the level of phosphoserine-121 CREB following UV or hydroxyurea exposure. Moreover, UV light triggered CREB serine-121 phosphorylation was strongly suppressed or not detectable in cells transfected with siRNA against ATM or in ATM-deficient cells, while ATR siRNA only partially suppressed CREB phosphorylation in response to UV light. These observations indicate that ATM is the major kinase that mediates CREB phosphorylation, while the role of ATR as

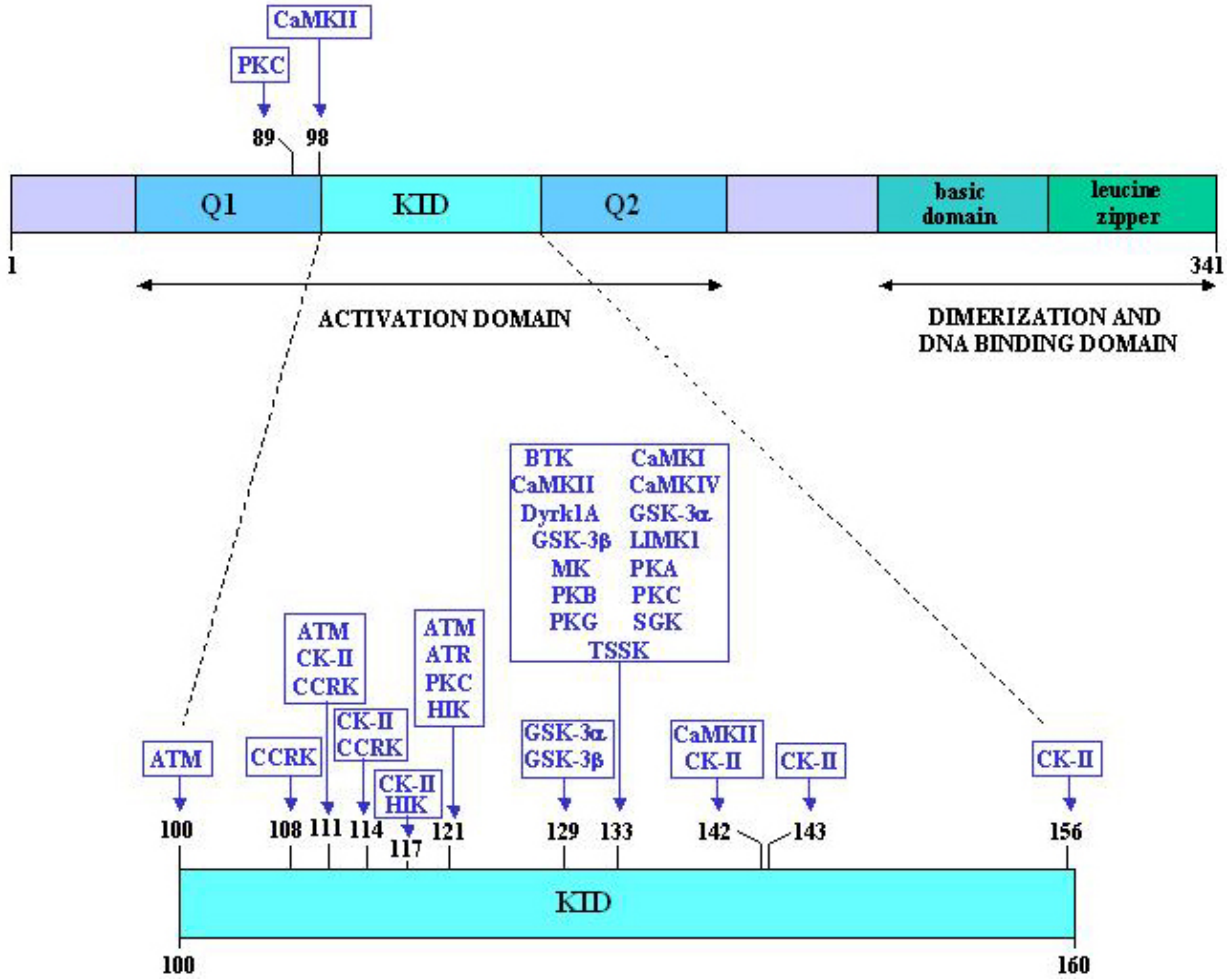

Figure 1. Schematic presentation of CREB and the known protein kinases that mediate CREB phosphorylation. The glutamine rich domains Q1 and Q2 and the kinase-inducible domain (KID) constitute the transcription activation domain of CREB, while the basic region and the leucine zipper domain form the dimerization and DNA-binding region of the protein. The lower part of the figure shows an enlargement of the KID. The phosphoacceptor sites and the protein kinases that mediate CREB phosphorylation at these sites are shown. Threonine 119, which was found to be phosphorylated by an unidentified protein kinase in a cell-cycle-dependent manner is not indicated (23). ATM=Ataxia-telangiectasia mutated; ATR=ATM-Rad3-related kinase; bFIK=bFGF-inducible kinase; BTK=Bruton's tyrosine kinase; CaMK= Ca^{2+}/calmodulin kinase; CKII=casein kinase 2 or protein kinase CK2; GSK3=Glycogen synthase kinase-3; HIP=hypoxia-induced kinase(s); LIMK1=LIM kinase 1; MK=Mitogen-activated protein kinase activated protein kinases; PKA=cAMP-dependent protein kinase; PKB=protein kinase B; PKC=protein kinase C; PKG=cGMP-dependent protein kinase; SGK=serum/glucocorticoid-inducible kinase; TSSK5=testis-specific serine/threonine kinase 5.

an *in vivo* CREB kinase is jeopardized (13). The functional implications of ATM-CREB pathway were not fully elucidated, but the authors speculated that this pathway might be involved in the transcription of specific CREB genes. Work by the group of Montminy has suggested that the PKA pathway preferentially activates CRE-containing promoters that posses a consensus TATA box (14). It is possible that ATM affects the expression of TATA-less CREB target genes, which include DNA repair factors and cell cycle regulators (12).

4.2. Protein kinase CK2 or casein kinase II (CK-II)

Protein kinase CK2 or casein kinase II is a tetrameric complex consisting of two catalytic subunits and two regulatory subunits. In humans two different forms of the catalytic subunits exists, designated alpha and alpha', while only one form of the regulatory subunit (referred to as beta) has been described. Recently, a third catalytic isoform alpha'' has been reported, which is almost identical to alpha except for the C-terminal domain. More than 300 potential targets for protein kinase CK2 have been described, including several transcription factors (15-17). One potential transcriptional factor target is CREB. It was originally shown that CK2 phosphorylated CREB *in vitro* at an unknown residue distinct from serine-133 (18). Subsequent *in vitro* protein kinase assays showed that CK2 phosphorylated CREB at two sites within the domain encompassing residues 106-121. This region

Table 1. Protein kinases that can mediate phosphorylation of CREB and the biological effects of these phosphorylation events

Residue	Protein kinase	Effect Of Phosphorylation On CREB	References
S-89	PKC	not tested	23
S-98	CaMK-II	not tested	57
T-100	ATM, ATR	KID-KIX interaction ↓[1]; transcriptional activity ↓	12
S-108	CK2	not tested	23
S-111	ATM, ATR	KID-KIX interaction ↓; transcriptional activity ↓	12
S-111	CK2	transcriptional activity ↓	20
S-111	CK2	transcriptional activity ↑	23
S-114	CK2	transcriptional activity ↓	20
S-114	CK2	transcriptional activity ↑	23
S-117	CK2	transcriptional activity ↓	20,23
S-117	HIK	ubiquitination/proteasomal degradation	100
T-119	CK2	not tested	23
S-121	ATM, ATR	KID-KIX interaction ↓; transcriptional activity ↓; ubiquitination/proteasomal degradation	12
S-121	HIK	not tested	100
S-121	PKC	dimerization ↑	55
S-129	GSK-3alpha and -3beta	DNA binding ↓	19,95-98
S-129	GSK-3alpha and -3beta	transcriptional activity ↑	21,94
S-129	GSK-3alpha and -3beta	CREB-CBP interaction ↓; transcriptional activity ↓	98
S-133	bFIK	transcriptional activity ↑	108
S-133	BTK	transcriptional activity ↑	106
S-133	CaMK-I	transcriptional activity ↑	77,81
S-133	CaMK-II	transcriptional activity ↑; transcriptional activity ↓	18,83
S-133	CaMK-IV	transcriptional activity ↑	83
S-133	DYRK1A	transcriptional activity ↑	29
S-133	HIK	not tested	96,101
S-133	LIMK-1	transcriptional activity ↑	27
S-133	MK2	transcriptional activity ↑	42
S-133	MK3	transcriptional activity ↑	47
S-133	MSK1	not tested	34,40
S-133	MSK2 (RSK-B)	transcriptional activity ↑	39,40
S-133	$p70^{SK6}$	not tested	50,54
S-133	PKA	transcriptional activity ↑	56
S-133	PKB	transcriptional activity ↑	62
S-133	PKC	transcriptional activity =	55,69
S-133	PKG	not tested	71
S-133	RSK1	transcriptional activity ↑	46
S-133	RSK2	transcriptional activity ↑	46,52
S-133	RSK3	transcriptional activity ↑	46
S-133	SGK	not tested	110
S-133	TSSK5	transcriptional activity ↑	25
S-142	CK2	not tested	22
S-142	CaMK-II	transcriptional activity ↓; dimerization ↓; CBP-binding ↓	82,83
S-142	CaMK-II	transcriptional activity ↑	18,87
S-143	CK2	not tested	22
S-156	CK2	transcriptional activity ↓	20

[1]The symbols indicate that transcriptional activity of CREB increases (↑), decreases (↓) or remains unaffected (=).

contains the putative CK2 phosphoacceptor residues serine-108, -111, -114, -117 and –121. The exact identity of the phosphoacceptor sites was not determined (19). Another group performed tryptic mapping of *in vitro* phosphorylated CREB by CK2 and their results revealed phosphorylation of the serine residues 111, 114, 117, and 156. Mutation of any or all of these sites did not reduce but rather increased CREB activity somewhat in the presence of cAMP (20). CREB was also shown to be phosphorylated at the CK2 consensus

sites in unstimulated rat osteoblastic UMR106-01 cells, but the exact residues were not determined (21). In addition, CK2 phosphorylated CREB at serine-143 and to a lesser extent serine-142 *in vitro*, a modification that disrupts *in vitro* KID:KIX interaction (11,22). The biological relevance of these phosphorylations remains elusive.

In contrast to stimuli-induced phosphorylation, Saeki and co-workers reported a cell-cycle-dependent hyperphosphorylation in the early S phase in human amnion FL cells. Phosphorylation occurred at serines 108, 111, 114 and 117, and at threonine 119. Transfection studies with an expression vector for the alpha catalytic subunit of CK2, resulted in hyperphosphorylation of CREB, while mimicking phosphorylation by substituting serines 111 and 114 by glutamic acid resulted in a CREB mutant with enhanced transcriptional activity compared to wild-type CREB (23). A direct *in vivo* interaction between CREB and CK2 has so far not been detected, but the role of CK2 in cell cycle regulation is well documented (15,17). It is therefore tempting to speculate that CK2 can mediate some of its effects on the cell cycle through phosphorylation of CREB.

4.3. Testis-specific serine/threonine kinase 5

Five members have been identified so far in the human testis-specific serine/threonine kinase (TSSK) family. A sixth, non-functional kinase that lacks the complete kinase domain (TSSK1b) is encoded by a pseudogene. TSSK1-5 are predominantly expressed in the testis, but TSSK1 is also present in the pancreas, while TSSK2 is observed in heart, brain and placenta tissue as well (24). In a yeast two-hybrid screen of a human testis cDNA library, a clone encoding the KID region of CREB was isolated as an interaction partner with the bait TSSK5. *In vitro* GST-pull down assays confirmed the physical association between these two proteins, but their direct association in cells was not addressed. TSSK5, but not the kinase dead TSSK5 K54M mutant, was shown to phosphorylate CREB at serine-133 *in vitro* and to activate CRE-dependent transcription (25).

4.4. LIM kinase 1

The serine/threonine LIM kinase family comprises of the two members LIMK1 and LIMK2. These kinases consist of two N-terminal LIM domains and one PDZ domain, a proline-serine rich region and a C-terminal kinase domain. A shorter catalytically inactive LIMK1 variant, the LIMK(-)isoform, is produced by alternative splicing of exon 11 (26). LIMK1 is predominantly expressed in the neurons of adult mouse and human brains, but low levels of LIMK1 mRNA are also found in many other adult tissues. LIMK1 plays a role in the development and maintenance of neuronal circuits that mediate cognitive functions. Deletion of the *Limk1* gene seems to be implicated in Williams syndrome, a genetic disorder characterized by mental retardation or learning difficulties and extreme weakness in visuospatial constructive cognition. The activity of LIMK1 is regulated by phosphorylation of threonine-508 in the kinase domain. The p21-activated kinase (PAK1) can phosphorylate LIMK1 at this residue, while the Rac/Cdc42-mediated pathway can also activate LIMK1. In a yeast two-hybrid assay, LIMK1 was shown to interact with a CREB mutant in which the motif RRPSY, encompassing serine-133, was replaced by RRSLY. This modification is frequently used to enhance the interaction and to stabilize the complex formation of CREB with its kinase in yeast. Moreover, co-immunoprecipitation of rat embryonic hippocampal H19-7 cells treated with basic fibroblast growth factor (bFGF) revealed an interaction between endogenous LIMK1 and CREB. Activated LIMK1 triggered CREB phosphorylation at serine-133 *in vitro* and *in vivo* and stimulated CRE-dependent transcription. Ectopical expression of a kinase-deficient LIMK1 inhibited bFGF-induced CREB phosphorylation and attenuated neurite outgrowth. These results indicate that activated LIMK1 and subsequent CREB phosphorylation and activation may be implicated in the neuronal differentiation of central nervous system hippocampal progenitor cells (27).

4.5. DYRK1A

At least seven closely related homologous mammalian kinases have been isolated in the dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) family. These enzymes possess serine and threonine phosphorylation activity, as well as autophosphorylation activity on tyrosine. One of the best-characterized members is DYRK1A. The human and rodent *Dyrk1A* genes are ubiquitously expressed in adult and fetal tissues with high expression in the brain and the heart during development. The human gene is located on chromosome 21 (21q22.2), a region that maps within the Down syndrome critical region and DYRK1A is overexpressed in Down syndrome embryonic brain. This suggests that DYRK1A is implicated in the neurobiological alterations observed in Down syndrome patients. Overexpression of this kinase potentiates nerve growth factor-induced neuronal differentiation of PC12 cells, indicating a functional role for DYRK1A in neuronal differentiation processes. (28 and references therein). DYRK1 was shown to interact with CREB in a yeast two-hybrid assay and bFGF stimulation promoted complex formation between DYRK1 and CREB in H19-7 cells. Activated DYRK1 phosphorylated CREB, but not CREBS133A *in vitro*, indicating that serine-133 is the only target of DYRK1. Transient overexpression of the kinase-deficient DYRK1 K188R mutant strongly diminished bFGF-induced CREB phosphorylation and attenuated bFGF-stimulated CRE-dependent transcription of the luciferase reporter gene. All these observations suggest that DYRK1 is a genuine CREB kinase (29). A DYRK1A-interacting protein seems to control phosphorylation of CREB by DYRK1A. The cytoplasmic DYRK1A-interacting protein PAHX-AP1 (phytanoyl-CoA alpha-hydroxylase associated protein 1), also known as PHYHIP (phytanoyl-CoA alpha-hydroxylase interacting protein), is exclusively expressed in the brain. Overexpression of PAHX-AP1 in PC12 cells resulted in nuclear exclusion of DYRK1A and association with cytoplasmic PAHX-AP1. In accordance, no interaction between DYRK1A and CREB was observed in untreated PC12 cells. However, in cells exposed to nerve growth factor, complexes between

DYRK1A and phosphoCREB were detectable. Thus stimuli may govern the subcellular localization of DYRK1A and hence its ability to phosphorylate CREB (30).

DYRK3, another member of the DYRK family, triggered serine-133 phosphorylation and stimulated CRE-dependent and GAL4-CREB-dependent transcription. CREB phosphorylation and activation was independent of the kinase activity of DYRK3, but was blocked by the PKA inhibitor H-89. Activation of CREB by DYRK3 seems to be indirect as it was demonstrated that this kinase interacts with PKA, which results in activation of the CRE/CREB-pathway (31). No direct interaction between DYRK3 and CREB or *in vivo* phosphorylation of CREB by DYRK3 has been shown so far.

4.6. Mitogen-activated protein kinase activated protein kinases (MAPKAPK)

The mitogen-activated protein kinases (MAPK) regulate diverse cellular activities including gene expression, mitosis, metabolism, motility, cell survival, apoptosis, and differentiation. The MAPK pathways consist basically of a three-component module in which a cascade of phosphorylation events successively activates a MAP kinase kinase kinase (MKKK), a MAP kinase kinase (MKK), and a MAP kinase (MAPK). MAPK can phosphorylate substrates directly, or may phosphorylate yet another kinase (MAPK activated protein kinase or MAPKAPK or MK), which then phosphorylates substrates (32).

4.6.1. MSK1 and MSK2 (=RSK-B)

Early studies by the group of Greenberg led to the identification of a nerve growth factor (NGF)-inducible CREB Ser-133 kinase in PC12 cells that could phosphorylate CREB *in vitro*. This protein kinase had an apparent molecular mass of 105 kDa and was immunologically distinct from the ~90 kDa RSK or MAPKAPK1. The true identity of this novel kinase was not established at that moment (33). In 1998 two novel MAPKAPK were isolated that were activated by the MEK/ERK as well as the MAPK p38 pathway and were referred to as mitogen- and stress-activated protein kinases-1 and –2 (MSK1 and MSK2). MSK1 phosphorylated CREB only at serine-133 *in vitro* and the synthetic peptide CREBtide (=peptide corresponding to amino acids 123-136 of CREB) also formed an extremely good substrate. The rate of CREB phosphorylation by MSK1 was 30-fold higher than RSK1, 12-fold higher than RSK2 and 60-fold higher than MK2. CREB phosphorylation at serine-133 in NGF-treated PC12 cells correlated well with the activation of MSK1, but not RSK1 nor MK2. This suggests that MSK1 rather than RSK1 or MK2 catalyzes NGF-triggered phosphorylation of CREB (34). Another study demonstrated that anisomycin-induced phosphorylation of CREB coincided with increased activity of MK2 and MSK-1 and was completely blocked by the MAP kinase p38 inhibitor SB203580. However, in anisomycin-treated MK2$^{-/-}$ fibroblasts, phosphoserine-133 CREB levels were comparable to wild-type cells. This suggests that MSK1 rather than MK2 mediates anisomycin-triggered CREB phosphorylation (35). Additional indications that MSK1 is a CREB kinase comes from the observations that MSK1 phosphorylates serine-133 *in vitro*, that the potent MAPKAPK1 and MSK1 inhibitor Ro31822 prevented mitogen-triggered CREB phosphorylation, and that MSK1 resides in the nuclei of both unstimulated and stimulated cells. Moreover, phorbol ester TPA- and epidermal growth factor (EGF)-induced phosphorylation of CREB at serine-133 in mouse embryonic stem cells was strongly reduced in MSK1$^{-/-}$ cells compared to wild-type cells, while brain derived growth factor-induced CREB phosphorylation in neurons was abolished in neurons deficient for MSK1, but not in MSK2$^{-/-}$ neurons (36,37).

UVC irradiation or EGF treatment of HeLa cells provoked readily phosphorylation of CREB at serine-133 and induced the activity of a ~108 kDa protein that could phosphorylate the CREBtide *in vitro*. UVC-induced activation of this kinase was sensible to the MAPK p38 inhibitor SB203580 (38). The fact that both growth factors and stress activated this protein kinase, combined with the observation that the protein is activated in a p38-dependent manner indicate that MSK1 represents the protein kinase of 108 kDa described by Iordanov and co-workers or the ~105 kDa CREB kinase isolated by Ginty and colleagues (33,34,38). Alternatively, MSK2, also known as RSK-B, may be the involved CREB kinase. MSK2 is an approximately 100 kDa protein kinase that is widely expressed and that phosphorylates CREBtide in an *in vitro* in-gel assay. Moreover, MSK2 stimulated CRE- and GAL4-CREB-dependent transcription and resides in the nucleus (39). Studies in primary embryonic fibroblasts derived from *Msk1*$^{-/-}$, *Msk2*$^{-/-}$ or double knock-out mice affirmed MSK1 and MSK2 as the major protein kinases that phosphorylate CREB at serine-133 in response to mitogens and cellular stress (40).

4.6.2. MK2 (MAPKAPK-2)

MK2 was shown to phosphorylate CREB *in vitro* (41), and several studies have identified MK2 as an *in vivo* CREB kinase. Tan and co-workers suggested that MK2 is the CREB kinase that phosphorylates serine-133 in human neuroblastoma cells SK-N-MC, primary astrocytes and L6 myocytes exposed to bFGF. Their conclusion is based upon following observations: *i.* MK2 phosphorylated CREB *in vitro*; *ii.* bFGF stimulated MK2 kinase activity; *iii.* inhibition of the upstream activator of MK2, MAPK p38, by SB203580 prevented bFGF-induced CREB phosphorylation and *iv.* MK2 immunoreactivity was detected in the MonoQ fraction that phosphorylated the synthetic substrates CREBtide and MK2tide (KKLNRTLSVA). Immunoreactive proteins of ~55 and ~45 kDa were detected (42). These may represent two isoforms of MK2 or cross-reactivity with closely related MK (43-45). The role of MK2 as a CREB kinase was also illustrated in NGF-induced serine-133 phosphorylation of CREB. NGF treatment resulted in MAPK p38-dependent activation MK2 and activated MK2 phosphorylated CREBtide *in vitro* (46). UVC irradiation of HeLa cells also strongly activated MK2, but the immunoprecipitated MK2 poorly phosphorylated CREB. However, overexpression of

a dominant negative MK2 attenuated UV-induced activation of GAL4-CREB-dependent transcription (38).

4.6.3. MK3 (MAPKAPK-3)

CREB is constitutively phosphorylated at serine-133 during *in vivo* maturation of the rat corpus luteum. This phosphorylation pattern coincides with increase of MK3 mRNA and protein levels, while MK2 mRNA and protein levels diminished. Moreover, MK3 remained in an activated state during luteal maturation. Immunoprecipitated MK3 was able to phosphorylate CREB *in vitro*, while transient transfection studies in the mature granulose cell line GRMO2 revealed that co-expression of wild-type MK3, but not a kinase-deficient mutant stimulated GAL4-CREB-mediated transcription (47). Studies by others have confirmed that MK3 readily phosphorylates CREB *in vitro* (41,48). Moreover, stimulation of the *bcl*-2 promoter by insulin-like growth factor-I in PC12 cells involved p38 MAPK and CREB and was blocked by over-expression of a dominant negative MK3 mutant (49). All these results support a role for MK3 as CREB kinase.

4.6.4. RSK1 (MAPKAPK-1a)

Hepatocyte growth factor/scatter factor plus endothelin-1 stimulation of human melanocytes resulted in a robust increase in CREB serine-133 phosphorylation (50). In-gel kinase assays further showed that monocytes expressed two constitutively active kinases of ~40 kDa and ~80 kDa that phosphorylated GST-CREB. Interestingly, human melanocytes express high levels of constitutively active PKA and the 40 kDa corresponds well with the molecular sizes of the different catalytic subunit isoforms, suggesting that the constitutive active 40 kDa kinase represents the catalytic subunit of PKA. RSK1 activity was enhanced five fold in growth factor stimulated cells and immunoprecipitated RSK1 phosphorylated CREB *in vitro*. Moreover the upstream activator of RSK1 (MEK1/2) was activated in growth factor stimulated melanocytes. This indicates that the inducible ~100 kDa kinase is RSK1 (50).

4.6.5. RSK2 (MAPKAPK-1b)

Xing and co-workers purified to homogeneity a CREB kinase from TPA-treated human erythroleukemia K562 cells. Peptide sequencing identified this protein kinase as RSK2. The same kinase was also purified from NGF-stimulated PC12 cells and EGF enhanced the kinase activity of RSK2. Cotransfection of GAL4-CREB and HA-tagged RSK2 in COS cells provoked GAL4-CREB phosphorylation at serine-133 when cells were treated with TPA or EGF. The authors did not report coimmunoprecipitation of the two ectopic expressed proteins, but purified RSK2 phosphorylated baculovirus expressed CREB at serine-133 *in vitro* (51). These observations strongly indicate that RSK2 is a genuine CREB kinase. In a follow-up study, the same group showed that NGF also activated RSK1 and RSK3 and that they could mediate CREB phosphorylation at serine-133 *in vivo* (46).

The role of RSK2 as a growth factor-induced CREB kinase was also seen in a fibroblast cell line established from a patient with Coffin-Lowry syndrome. Coffin-Lowry syndrome is X-linked disorder characterized by skeletal deformation and mental retardation. All patients suffering from this syndrome have been shown to possess mutations in the *Rsk*-2 gene, which impair the kinase activity of the enzyme. Phosphorylation of CREB at serine-133 and induction of the CREB target gene c-*fos* were severely attenuated in an EGF-treated fibroblast cell line established from a patient with Coffin-Lowry syndrome compared to treated control cells (52). However, contradictory results were obtained by other investigators who detected CREB phosphorylation in response to EGF, as well as TPA in immortalized fibroblasts from Coffin-Lowry syndrome (40). Moreover, Brüning *et al*. showed that platelet-derived growth factor as well as insulin-like growth factor stimulated CREB serine-133 phosphorylation in immortalized embryonic fibroblasts generated from *Rsk2*$^{-/-}$ mice with the same kinetics and stoichiometry as in wild-type cells and CREB-dependent transcription after stimulation was comparable in knockout and wild-type cells (53) These discrepancies of RSK as a growth factor-induced CREB kinase may be due to use of different stimuli and/or cell systems .

4.6.6. p70^{SK6}

Treatment of human melanocytes with hepatocyte growth factor/scatter factor plus endothelin-1 vividly triggered CREB serine-133 phosphorylation and increased p70^{SK6} kinase activity 1.7 times. Immunoprecipitated p70^{SK6} from growth factor treated melanocytes was able to phosphorylate GST-CREB *in vitro*. However, inhibition of p70^{SK6} by the selective inhibitor rapamycin had no effect on growth factor-provoked CREB phosphorylation, indicating that p70^{SK6} is not the major CREB kinase under these experimental conditions (50). Bacterially expressed CREB is efficiently phosphorylated *in vitro* by p70^{SK6} purified from the liver of cycloheximide-treated rats. It was not tested whether serine-133 was the target, but the corresponding serine-117 of CREMtau was the unique residue for phosphorylation by p70^{SK6} (54). Although these results suggest that p70^{SK6} can act as a CREB kinase, proof that this kinase is a *bona fide* CREB kinase is lacking.

4.7. PKA

As CREB mediates cAMP-induced transcription and because the primary sequence of the CREB protein contains a putative PKA site at serine-133, it was not unforeseen that cAMP-dependent protein kinase or PKA was the first CREB kinase to be identified. Tryptic peptide mapping of *in vitro* phosphorylated CREB revealed that PKA phosphorylated exclusively at serine residue 133 (55,56). In another *in vitro* study, it was found that the purified catalytic subunit of PKA was able to phosphorylate CREB at both serine-98 and serine-133. However, serine-98 was only phosphorylated at relative high concentrations of PKA (PKA:CREB molar ratio of 1:3) and studies with GAL4-CREB fusion proteins revealed that mutation of serine-98 into alanine had no effect on basal or PKA-induced CREB-mediated transcription. Hence, PKA mediated phosphorylation of serine-98 most probably represents an *in vitro* artifact. A functional role of this residue may, however, not be excluded as this amino acid forms the phosphoacceptor site in the CaMKII consensus

motif (57). Indications that PKA may phosphorylate CREB at serine-133 directly *in vivo* come from following observations. Microinjection of the catalytic subunit activated CRE-dependent transcription, while forskolin-induced activation of the PKA signaling pathway provoked CREB phosphorylation in PC12 cells, but not in PKA-deficient PC12 cell lines (56,58,59). Specific inhibitors of PKA can inhibit *in vivo* serine-133 phosphorylation (reviewed in 9). Mutagenesis of the basic arginine residues within the consensus PKA site (RRXS; RRFS in CREB) renders CREB inactive, underscoring that PKA directly phosphorylates without any cascade intermediates (60).

4.8. PKB

Akt/PKB protein kinase is a serine/threonine kinase that initially was identified by three independent groups, based on its homology to PKA and PKC or as the cellular homolog to the retroviral oncogene viral akt (v-*akt*). Three Akt/PKB genes have been identified in mammalian, termed Akt1/PKBalpha, Akt2/PKBbeta and Akt3/PKBgamma. All three isoforms consist of a conserved domain structure, consisting of an amino terminal pleckstrin homology (PH) domain, a central kinase domain and a carboxyl-terminal regulatory domain. The Akt/PKB protein kinase is a critical regulator of cell survival and proliferation. One mechanism by which PKB exerts its biological functions may involve phosphorylation of transcriptional factors like for example CREB (61). Indeed, the group of Montminy showed that PKB might be a CREB kinase. Immunoprecipitations of wild-type Akt/PKB and kinase dead Akt/PKB were performed from serum stimulated 293T cells. Wildtype Akt/PKB, but not kinase dead Akt/ PKB, was shown to phosphorylate CREB Ser-133 by *in vitro* kinase assays. Mammalian two-hybrid studies revealed that PKB stimulated recruitment of CBP to CREB, and Akt/PKB enhanced CRE-driven reporter gene activity. PI3K-mediated activation of Akt/PKB was also shown to result in increased GAL4-CREB activity *in vivo* via a Ser-133 dependent mechanism (62).

4.9. PKC

The serine/threonine protein kinase C family consists of several members that are divided into three major groups: the classical PKC (alpha, beta and gamma), the novel PKC (delta, epsilon, eta and theta) and atypical PKC (lambda, zeta and iota). PKC activation occurs in response to various growth factors and results in distinct cellular responses, including differentiation, physiological processes, proliferation, apoptosis and migration. Different groups have shown that PKC isoforms phosphorylate CREB *in vitro* at Ser-133, while some groups reported two additional residues that have not been characterized yet. Serine-121 has been proposed as a PKC phosphoacceptor site, while a threonine represents the third target for phosphorylation (18,63,64). *In vitro* DNA binding studies showed increased dimeric CREB-DNA complexes with CREB that had been *in vitro* phosphorylated by PKC, but not by PKA. Thus phosphorylation at additional sites may correlate with increased dimerization of CREB (55,56).

Although purified PKC can phosphorylate CREB, it has not been determined whether PKC phosphorylates CREB directly *in vivo* or via PKC-activated protein kinase(s). Phosphorylation of CREB at serine-133 induced by signaling through the B cell antigen receptor requires PKCdelta (65). However, RSK1, rather than PKCdelta seems to mediate direct phosphorylation of CREB, suggesting that *in vivo* PKC seems to act upstream of other CREB kinases rather than to phosphorylate CREB directly. Phosphorylation of CREB and CREB-mediated transcription triggered in activated primary human T cells was inhibited by specific inhibition of PKCtheta. A direct involvement of this PKC isoform as a CREB kinase is lacking (66). PKCepsilon has also been implicated in cytokine-induced serine-133 phosphorylation and activation of CREB-mediated transcription in the human erythroleukemia cell line TF-1, but again PKCepsilon forms a component of the signal cascade rather than acting as the genuine CREB kinase. However, indirect evidence may suggest that PKCepsilon directly contributes to CREB phosphorylation. Specific inhibition of PKC completely prevented, while blocking the MEK/ERK pathway attenuated cytokine-induced CREB phosphorylation (67). Moreover, PKCepsilon has been insinuated to phosphorylate CREB *in vitro* (68).

Cotransfection studies with a CRE-CAT reporter plasmid, an expression vector for the catalytic subunit of PKA and CREB expression vectors showed that deletion of amino acids 121-125, which contains a putative PKC site with serine-121 as the phosphoacceptor site, reduced CREB-mediated transcription by 75% compared to wild-type CREB. The inducibility of this mutant was about half of that of wild-type CREB. This indicates that serine-121 may be target for other kinases and that mutation of this site affects PKA-induced CREB-mediated transcription (60).

Cotransfection with an expression vector encoding constitutively active PKCalpha did not induce GAL4-CREB-mediated transcription or CRE-dependent promoter activity in Jurkat cells (69). In contrast with this, antisense-mediated suppression of PKCalpha expression, as well as decreased nuclear PKCalpha levels in liver during sepsis resulted in reduced phosphoCREB levels, suggesting that PKCalpha can mediate CREB phosphorylation in these cells (70). Ectopic expression of a constitutive active mutant of PKCzeta failed to stimulate the transcriptional activity of GAL4-CREB in transient transfection studies in NIH3T3 cells (our unpublished results). This result excludes a role for this PKC isoform in the regulation of CREB activity. In conclusion, despite the overwhelming reports that specific PKC inhibitors may abrogate stimuli-induced CREB phosphorylation (see supplementary data in 9), proof for PKC as a genuine CREB kinase is still lacking.

4.10. PKG

Two different genes encode the soluble cyclic GMP (cGMP)-dependent protein kinase I (or PKG I) and membrane-bound PKG II. In addition, two different splice variants of PKG I have been described. PKG I is widely expressed, while PKG II shows a more limited expression pattern. PKG´s kinase activity is regulated by cGMP.

cGMP can be generated by two different pathways: (i) by cytoplasmic, soluble guanylate cyclases (sGCs) that are activated by nitric oxide (NO) and carbon monoxide, or (ii) by receptor guanylate cyclases (rGCs) that are activated by natriuretic peptides and guanylins. Increased intracellular cGMP leads to increased CREB Ser-133 phosphorylation in vascular smooth muscle cells, neuronal cells, and PKG transfected Baby Hamster Kidney (BHK) cells, but not in PKG-deficient BHK cells (71). This suggests a direct or indirect involvement of PKG in CREB phosphorylation. Gudi and colleagues found that increased intracellular cGMP induced nuclear translocation of PKG I with a kinetic that correlated with CREB Ser-133 phosphorylation. The cGMP-induced CREB phosphorylation occurred independently of changes in intracellular Ca^{2+} concentrations, activation of MAP-kinases (ERK1/2 and p38) thus ruling out the effect of other known CREB kinases (72). Other groups have also demonstrated the involvement of NO, cGMP and PKG I in phosphorylation of CREB (reviewed in 71). However, neither of the studies showed direct interaction between CREB and PKG. Thereto, different groups have investigated whether PKG could phosphorylate CREB directly *in vitro*. An early study revealed that PKG phosphorylated CREB at a slower rate than PKA (73). Another group failed to detect *in vitro* phosphorylation when immunoprecipitated CREB from primary cultures of olfactory receptor neurons were incubated with PKG purified from either rat brain or bovine lung (74). Gudi and colleagues, using another experimental approach, demonstrated that GSTCREB fusion protein isolated from transfected BHK cells as well as bacterial expressed GSTCREB were phosphorylated by both PKA and PKG *in vitro* (reviewed in 71). Therefore, the role of PKG as a CREB kinase remains controversial.

4.11. Ca^{2+}/calmodulin-dependent kinases I, II, and IV

As a result of increased intracellular calcium concentrations, Ca^{2+} can associate with a Ca^{2+}-binding protein known as calmodulin. Ca^{2+}/calmodulin complexes can interact and activate a variety of cellular effectors, including the Ca^{2+}/calmodulin-dependent kinases CaMK. Three family members of CaMK have been described, type I, II and IV. CaMKII is a multimeric enzyme composed of at least 10 subunits. Four types of CaMKII subunits have been identified (alpha, beta, gamma and delta), which are encoded by four different genes. Moreover, isoforms due to alternative splicing exist. CaMKII is activated through transphosphorylation of the subunits, while the monomeric CaMKI and CaMKIV enzymes are activated by CaMK kinases. CaMKIV is predominantly nuclear, while CaMKI appears to be a cytosolic enzyme. The subcellular localization of CaMKII varies. Most isoforms are cytoplasmic, but some splice variants of the subunits contain nuclear localization signals that can direct CaMKII to this cell compartment (reviewed in 75).

Numerous studies have shown that constitutive active CaMKI, CaMKII, and CaMKIV phosphorylate CREB at serine-133 *in vitro*, while only CaMKI and CaMKIV were able to stimulate CREB-mediated transcription in a serine-133 dependent manner in different cell lines, including GH_3, JEG-3, PC12 and GC pituitary cells (59,76-82). The differential activation of CREB by CaMKI and CaMKII compared to CaMKIV results from differential phosphorylation. CaMKI and CaMKIV phosphorylated CREB directly at serine-133 *in vitro*, while CaMKII (both the alpha and the beta isoforms) phosphorylated both serine-133 and serine-142 with the same stoichiometry (79,82). The observation that CaMKII failed to induce CREB-mediated transcription indicates that phosphorylation of serine-142 exerts a negative effect on the transcriptional activity of CREB. This effect is also dominant over PKA-triggered phosphorylation of CREB as ectopic expression of a constitutive active CaMKII variant blocked PKA-triggered CREB activation. Moreover, CaMKII may also counteract the stimulation of the transcriptional potentials of CREB that occurs after unique phosphorylation of serine-133 by other protein kinases (83). The mechanism for the negative effect on CREB-mediated transcription due to phosphoserine-142 remains elusive. Phosphorylation of serine-142 did not have a detectable effect on DNA binding, nor did it interfere with PKA-induced interaction with CBP (18,83,84). However, another study demonstrated that modification of this residue by CaMKII prevented full length CREB dimerization and CBP recruitment *in vitro* (82). Moreover, previous studies had illustrated that protein-protein association with the KID and KIX domain fragments was ablated by phosphorylation of serine-142 because this modification likely disrupted the crucial interaction with tyrosine-650 in the KIX domain of CBP (11). These results of CaMKII-mediated modifications of CREB indicate that phosphorylation of serine-133 is not sufficient for recruitment of CBP, but that dimerization is also required (82). Using chromatin immunoprecipitation assays, however, it was shown that CaMKII-mediated CREB phosphorylation at serine-142 did not preclude CBP binding to CREB at the CRE of the c-*fos* promoter (85). The mechanism of CaMKII-mediated inhibition of CREB-dependent transcription may be even more complex as it has been demonstrated that monomeric CREB could activate transcription. However, these results are based on assays monitoring CREB-mediated transcription of a reporter gene with CREB fused to the DNA-binding domain of the B-cell specific activator protein BSAP that binds to DNA as a monomer (86) The results should be interpreted carefully because the experimental conditions may vary from the *in vivo* situation.

CaMKII-mediated dual phosphorylation of CREB could also activate CREB-directed transcription. Dash and colleagues observed that CaMKII increased the transcriptional potentials of CREB as monitored in an *in vitro* transcription assays with the CRE-containing c-*fos* promoter. Phosphorylation by CaMKII plus PKA increased transcription additively compared to phosphorylation by either one alone (18). Recently, another example of positive effect of ser-142 phosphorylation on CREB-mediated transcription was recognized. Membrane depolarization induced the phosphorylation of CREB at serines-133, 142 and 143 in cortical neuron cell cultures, as well as in neurons of rat brains. The phosphorylation of the different serine residues occurred with different kinetics. Serine-133

became phosphorylated within 1-2 minutes and sustained for at least 1 hour, while phosphorylation of serine-142 was only detectable after 5 minutes and started to decline after 30 minutes. Serine-143 was slightly later and decreased after 30 minutes. The differences in phosphorylation kinetics may suggest the involvement of distinct protein kinases, but their *in vivo* identities remain unknown. A nuclear isoform of CaMKII (alpha, delta, or gamma) is a likely candidate to mediate serine-142 phosphorylation, while CK2 may be responsible for phosphorylating serine-143. Membrane depolarization-induced phosphorylation of serine-142 and –143 led to an enhancement of GAL4-CREB-driven transcription as substitution of these residues into alanine attenuated CREB activity in response to membrane depolarization. However, these phosphorylation events disrupted *in vitro* phosphoserine-133 CREB:KIX interactions, indicating that this triple phosphorylated CREB mediates transcription through a mechanism distinct from the classical phosphoserine-133 CREB-CBP model as outlined in the introduction. Another puzzling observation was that replacing serine-142 by alanine in full length CREB enhanced GAL4-CREB-mediated transcription, while the same mutation in the context of CREB lacking the basic leucine zipper dimerization domain inhibited CREB activity (22). An additional elegant study confirmed a positive contribution of phosphoserine-142 in CREB-mediated transcription. Using a phosphoserine-142 specific antibody, Gau and collaegues showed that phosphorylation of serine-142 in cells of the suprachiasmatic nucleus (SCN) parallels the circadian oscillation of serine-133 phosphorylation. Light stimuli, as well as glutamate induced phosphorylation of both serine-133 and –142 and phosphorylation of serine-142 seemed to be required for the expression of c-*fos* and m*Per*1 genes because their expression was significantly attenuated in SCN cells of mouse encoding mutant CREB in which serine-142 was replaced by alanine. This study indicates that serine-142 is an *in vivo* phosphoacceptor site and phosphorylation of this site enhances the transcriptional activation of CREB. Although Gau *et al.* did not identify the protein kinase responsible for *in vivo* phosphorylation of serine-142, they speculated that CaMKII could be the CREB kinase that mediated dual phosphorylation of CREB in SCN cells during the circadian cycle (87).

What is the biological relevance of serine-142 phosphorylation by CaMKII? The observation that serine-142 phosphorylation can result in both positive and negative effects on CREB-mediated transcription may be a result of different stimuli and/or cell-types. This may create an opportunity to differententially express CREB responsive genes depending on the cellular context. Moreover, the time-dependent phosphorylation pattern between serine 133 phosphorylation versus serine 142 phosphorylation rises the possibility of differentiation between genes that require serine 133 phosphorylation only or those that depent on dual phoshorylation for their CREB-directed transcription. Serine-142 phosphorylation by CaMKII may also counteract activating serine-133 phosphorylation by other CREB kinases. Thus an additional phosphorylation event may offer an alternative to dephosphorylation of serine-133 to down-regulate the activity of CREB. It is possible that CREB is not a physiological substrate for CaMKII? While, CaMKIV is nuclear, CaMKII seems to reside predominatly cytoplasmic thus physically separating it from its substrate CREB. However, some CaMK-II isoforms (alpha, deltaB, or gamma) contain a nuclear localization signal, which is sufficient to localize these splice variants to the nucleus. Moreover, a proteolytic CaMKII variant that can reside in the nuclues has also been identified (see discussion in 83). The nuclear presence of CaMKII isoforms underscores a putative role for them in the regulation of CREB activity.

Several studies support a role for CaMKIV as a physiologically relevant CREB kinase. First, increase in levels of phosphoser-133 CREB measured in the spinal cord of wild-type mice after chronic morphine administration was absent in CaMKIV knockout mice (88). Second, cotransfection studies in COS7 cells using a CRE-luciferase reporter construct and constitutively active forms of CaMKIV and CaMK kinase gave an ~5-fold increase in luciferase activity, while this activation was blocked by dnCaMKIV, as well as the CREBS133A mutant. Finally, several groups have shown that Ca^{2+}-induced CREB phosphorylation and CREB-dependent gene expression were strongly impaired in CaMKIV deficient mice and in transgenic mice overexpressing dnCaMKIV (89-91).

Recently, a granulocyte-specific Ca^{2+}/calmodulin-dependent kinase, CKLiK, was isolated that is activated by interleukin-8. CKLiK can phosphorylate CREM at serine-117 *in vitro* and activates GAL4-CREB-directed transcription in a serine-133 dependent manner. Whether CKLiK could phosphorylate CREB was not tested, but interleukin-8 induced phosphorylation of CREB at serine-133, indicating that CKLiK can mediate phosphorylation of CREB in response to this cytokine (92).

4.12. Glycogen synthase kinase-3

Glycogen synthase kinase-3 (GSK3) was originally identified as a protein kinase involved in the regulation of glucose metabolism, but it is now recognized that GSK3 is also implicated in other cellular processes such as cell migration, cellular architectural dynamics, cell differentiation, cell survival and cell death. GSK3 exists in two isoforms, GSK3alpha and 3beta that are encoded by different genes, while a splice variant for GSK3beta has also been described. The different GSK3 isoforms have nearly identical sequences in their kinase domains. More than 40 proteins are substrates for GSK, including several transcription factors. The activity of GSK itself is regulated by (auto)phosphorylation, subcellular localization and interaction with other proteins. Phosphorylation of serine-9 (respectively serine-21) results in inactivation of GSK3alpha (respectively GSK3beta), while phosphorylation of tyrosine-279 (GSK3alpha) or tyrosine-216 (GSK3beta) enhances the enzymatic activity. PKA, PKB, PKC and RSK-1 have been shown to phosphorylate serine-9 and serine-21, while the protein kinases mediating tyrosine phosphorylation have been poorly characterized, but autophosphorylation of these tyrosine residues by GSK has been reported (recently reviewed in 93).

Phosphorylation of substrates by GSK3 obeys a hierarchical order, i.e. pre-phosphorylation has to occur before GSK3 can additionally phosphorylate the substrate. Phosphorylation of serine-133 in the CREB motif SRRPS*Y (with S* representing phosphoserine-133) creates a consensus site (SXXXS*P) for GSK3 (94). *In vitro* studies revealed that phosphorylation of CREB at serine-133 by PKA induced a conformational change in CREB, resulting in an increase in positive charge along with enhanced DNA binding both at canonical and non-canonical CRE motifs. Progressive phosphorylation of serine-129 and another unidentified serine residue within the amino acids 106-121 by GSK3 caused a further increase in spherical shape and net surface, yet reversed the increase in DNA binding affinity in response to phosphorylation by PKA. This suggests that GSK-3 mediated phosphorylation has a negative effect on the binding of CREB to CRE motifs. Surprisingly, substitution of all the serines residues into non-phosphorable alanine reduced the DNA binding affinity 5-fold. The effect of GSK3-induced phosphorylation on the transcriptional activity of CREB was not approached in this study (19). Another study revealed that GSK3beta completely blocked increases in CREB DNA binding activity induced by growth factors and cAMP elevating agents (95).

Studies by Fiol and colleagues reported that either isoform of GSK3 could phosphorylate CREB at serine-129 *in vitro* but only after primary phosphorylation of serine-133 (94). The specific GSK3 inhibitor II blocked basal CREB serine-129 phosphorylation levels in cardiomyocytes, underscoring a role for GSK3 as a CREB kinase *in vivo* (96). It is not known, however, which isoform(s) of GSK3 carry out serine-129 phosphorylation *in vivo*. GSK3alpha- and GSK3beta-mediated phosphorylation of serine-129 potentiated forskolin-induced activation of GAL4-CREB-dependent transcription in PC12 cells. Substituting serine-129 by alanine strongly reduced GSK3-triggered activation of GAL4-CREB-driven transcription in forskolin treated cells. Co-expression of GSK3beta strongly potentiated PKA- or forskolin-induced activation of the CREB responsive somatostatin promoter. These studies imply a functional role for GSK3 as a CREB kinase that stimulates phosphoserine-133 CREB-mediated transcription (94). In accordance with these findings, Tyson and co-workers presented evidence that GSK3beta-mediated phosphorylation of serine-129, which was required for the increased transcriptional activity of CREB in rat osteoblast UMR106-01 cells in response to parathyroid hormone (PTH). The exact mechanism by which phosphorylation of CREB at this position stimulated the transcriptional activity of CREB remains unknown, but no increased CREB binding to the c-*fos* CRE motif was observed in treated cells compared to unstimulated cells. Mimicking phosphorylation by replacing serine-129 by aspartic acid was sufficient to restore PTH-triggered CREB-mediated transcription when GSK3beta was inhibited. This suggests that a negative charge at this residue is required for increased CREB activity (21). These examples underscore a positive role of Ser-129 phosphorylation on CREB-mediated transcription. On the other hand, GSK3beta reduced cAMP-induced activation of the CREB targeted cholecystokinin promoter in SK-N-MC and PC12 cells. GSK3beta decreased *in vitro* binding of CREB to the cholecystokinin CRE motif, but had no effect on GAL4-CREB-mediated transcription. These findings strongly suggest that the inhibitory effect of GSK3beta on cAMP-enhanced cholecystokinin promoter activity resulted from diminished CREB binding to the promoter rather than down-regulation of the transcriptional activity of CREB (97). Inhibition of GSK3 in LPS stimulated monocytes also resulted in higher *in vitro* CRE binding activity, augmented the association of CREB with CBP and enhanced LPS-triggered expression of the CREB target gene IL-10 (98). Overall, these results points to a dual negative role of GSK3 in CREB-mediated transcription: inhibition of CREB DNA binding and prevention of CBP recruitment by phosphoserine-133 CREB.

Finally, GSK3beta may also modulate CREB-mediated transcription indirectly as it was recently shown that GSK3beta increased PP1-mediated dephosphorylation of phosphoserine-133 CREB and antagonized CRE-dependent transcription in neurons (99).

4.13. Hypoxia-induced kinase(s)

Several laboratories have addressed the effect of hypoxia on the phosphorylation and transcriptional activity of CREB and their results point to a cell-specific response. Indeed, studies by Taylor and co-workers demonstrated that hypoxia treatment of T84 intestinal epithelial cells resulted in serine phosphorylation of CREB possible in the sequence DSVTDS spanning residues 116-121 which resembles the phosphorylation-dependent proteasomal degradation targeting sequence DS*UXXS* (with S*=phosphoserine residue, U=hydrophobic amino acid, and X= any amino acid). The authors proposed that hyperphosphorylation of CREB resulted from reduced protein phosphatase activity in hypoxia-exposed cells. Indeed, hypoxia treatment of T84 cells reduced the levels of protein phosphatase PP1gamma, a protein phosphatase known to dephosphorylate CREB (100). Alternatively, hypoxia-inducible kinase(s) may be responsible for mediating CREB phosphorylation at serine residues 117 and 121. These putative kinases remain unidentified and direct proof for phosphorylation of these residues is lacking.

Hypoxia treatment resulted in clearly possessed increased levels of phosphoserine-133 CREB and unaffected total CREB protein amounts in pheochromocytoma PC12 cells. CRE-dependent promoter activity augmented under these conditions, but despite phosphorylation of serine-133, GAL4-CREB-mediated transcription was unaffected (101). Other CRE-binding proteins may mediate stimulation of a CRE-containing promoter, explaining why hypoxia-triggered activation of such promoters occurs in the absence of hypoxia-provoked activation of CREB. Hypoxia also induced CREB Ser-133 phosphorylation in primary rat cardiomyocytes, while no changes in the total amount of CREB protein level were monitored. The authors measured reduced CREB serine-129 phosphorylation and GSK3beta activity, and increased DNA-binding properties of CREB after 6 hours of hypoxia

conditions. Moreover, hypoxia/reoxygenation (regime of 6 hours/24 hours) induced CRE-dependent transcription in PC12 cells, an effect that was completely abolished in cells cotransfected with the constitutive active GSK3betaS9A mutant. These results favor a role for GSK3beta as a hypoxia-induced kinase. However, pharmacological inhibition of GSK3beta did not completely abolish CREB serine-129 phosphorylation, suggesting that other protein kinases can modulate the phosphorylation status of this residue (96). Hypoxia activated PKB/AKT, but CREB serine-133 phosphorylation persisted in cells pretreated with inhibitors of the phosphatidylinositol 3-kinase/AKT, PKA, PKC, PKG, CaMK, RSK-2 and MK2. These results exclude these kinases as hypoxia-induced CREB serine-133 kinases (96,101-103). The cAMP levels are strongly reduced in hypoxia treated cells, indicating that PKA is unlikely to mediate hypoxia-provoked phosphorylation of CREB (104). Inhibition of the PKA (H89), MEK1/2 (PD98059) and PI3K/Akt (LY294002) pathways, however, blocked CREB DNA binding, while specific inhibition of GSK3beta down-regulated, but not completely abolished serine-129 phosphorylation after hypoxia/reoxygenation regimes (96). In conclusion, the phosphorylation pattern and transcriptional activity of CREB in response to hypoxia seems to be cell-specific. Moreover, hypoxia may modulate the DNA-binding properties of CREB. The true identity of the protein kinases that mediate hypoxia-triggered CREB phosphorylation have not been delineated completely, but GSK3beta seems to be involved.

4.14. Bruton's tyrosine kinase (BTK)

Bruton's tyrosine kinase (BTK) is a non-receptor tyrosine kinase belonging to a family that in addition contains the members Itk/Emt/Tsk, Bmx/Etk, and Tec. These proteins are characterized by a pleckstrin homology domain in their N-terminal region, which allows interaction with e.g. phosphoinositides, heterodimeric G-protein subunits and PKC isoforms. BTK display a restricted expression profile in a subset of B cells and in myeloid cells, but expression is also observed in brain, bone marrow, spleen, lymph nodes and fetal liver (105,106). Yeast two-hybrid assay identified CREB as an interaction partner for BTK. This interaction was also confirmed in extracts of H19-7 cells. BTK phosphorylates CREB at serine-133 in vitro and in vivo and stimulates CRE-dependent transcription. bFGF-induced neuronal differentiation of H19-7 cells was inhibited by overexpression of kinase-deficient BTK or siRNA-mediated silencing of BTK and coincided with reduced CREB phosphorylation. These observations implicate a role for CREB in BTK-triggered differentiation of these cells (106). BTK can activate the p38 MAP kinase and PKC isoforms (105). Both p38 MAP kinase and PKC have been shown to converge to CREB, so that BTK triggered CREB phosphorylation may also be indirectly mediated through these kinases. Tec, another BTK family member, was not identified as a CREB interaction partner in the yeast two-hybrid experiment (107). This suggests a substrate specificity for the different members of this family of non-receptor tyrosine kinase.

4. 15. bFGF-inducible kinase (bFIK)

Basic fibroblast growth factor (bFGF) treatment of H19-7 cells triggered phosphorylation of CREB at serine-133 and stimulated CRE-dependent transcription. The kinases DYRK1A, LIMK1 and BTK have been shown to mediate serine-133 phosphorylation in bFGF-treated H19-7 cells. In addition, an in vitro in-gel kinase assays revealed that bFGF greatly induced a ~120 kDa protein kinase, which phosphorylated CREB at serine-133. bFGF-induced CREB phosphorylation was insensitive to specific inhibition of the PKA, PKC, Raf/MEK, MAPK p38, PI3K, CaMK, and protein kinase CK2 pathways, but the identity of the bFGF inducible kinase remains elusive (108).

4.16. Serum and glucocorticoid inducible protein kinase (SGK)

Serum and glucocorticoid inducible protein kinase, SGK, was originally discovered as a novel protein kinase that was under acute transcriptional control by serum and glucocorticoids. Some studies refer to SGK as SGK1 because two other SGK isoforms have been uncovered, named SGK2 and SGK3, however, less is known about the two latter. SGK is a 50 kDa protein that is member of the AGC family of serine/threonine protein kinases, and the catalytic domain of SGK is 45-55% homologous to the catalytic domains of Akt/PKB, PKA, PKC-zeta and the rat $p70^{S6K}$ /$p85^{S6K}$ kinases. SGK is a conserved protein, and homologues exist in organisms like human, rat, mice, *Caenorhabilitis elegans* and *Saccaromyces cereviciae.* SGK is regulated at several levels. First, SGK can be regulated at transcriptional level with induced expression to an expanding set of hormonal and nonhormonal extracellular cues. Second, SGK is directly phosphorylated and activated by phosphoinositide-dependent kinase 1 (PDK1), which acts immediately downstream of phosphoinositide 3-kinase. Finally, the subcellular localization of SGK is controlled in a cell cycle-dependent mode, as well as by exposure to specific hormones and environmental stress stimuli (reviewed in 109).

SGK was recently shown to physically interact with CREB, as well as to phosphorylate CREB Ser-133 in vitro. Co-transfection experiments with increasing amount of constitutive active SGK resulted in a dose-dependent increase in the abundance of phospho-CREB with no change in the abundance of CREB itself. Moreover, induction of SGK by dexamethasone, a glucocorticoid receptor agonist, resulted in CREB phosphorylation, which could be inhibited by the presence of dominant negative SGK (110). Lee and co-workers showed that the phorbol ester TPA caused activation of ERK and that SGK is an in vivo downstream target of ERK in the rat hippocampus. Overexpression of a dominant negative mutant of SGK (SGK S78A) antagonized TPA-induced CREB phosphorylation (111). The observations in these two studies suggest that SGK can mediate CREB Ser-133 phosphorylation.

5. BIOLOGICAL IMPORTANCE OF THE EXISTENCE OF NUMEROUS CREB KINASES

Post-translational modification in the form of reversible phosphorylation is a crucial regulatory mechanism for the activity of many proteins. Central

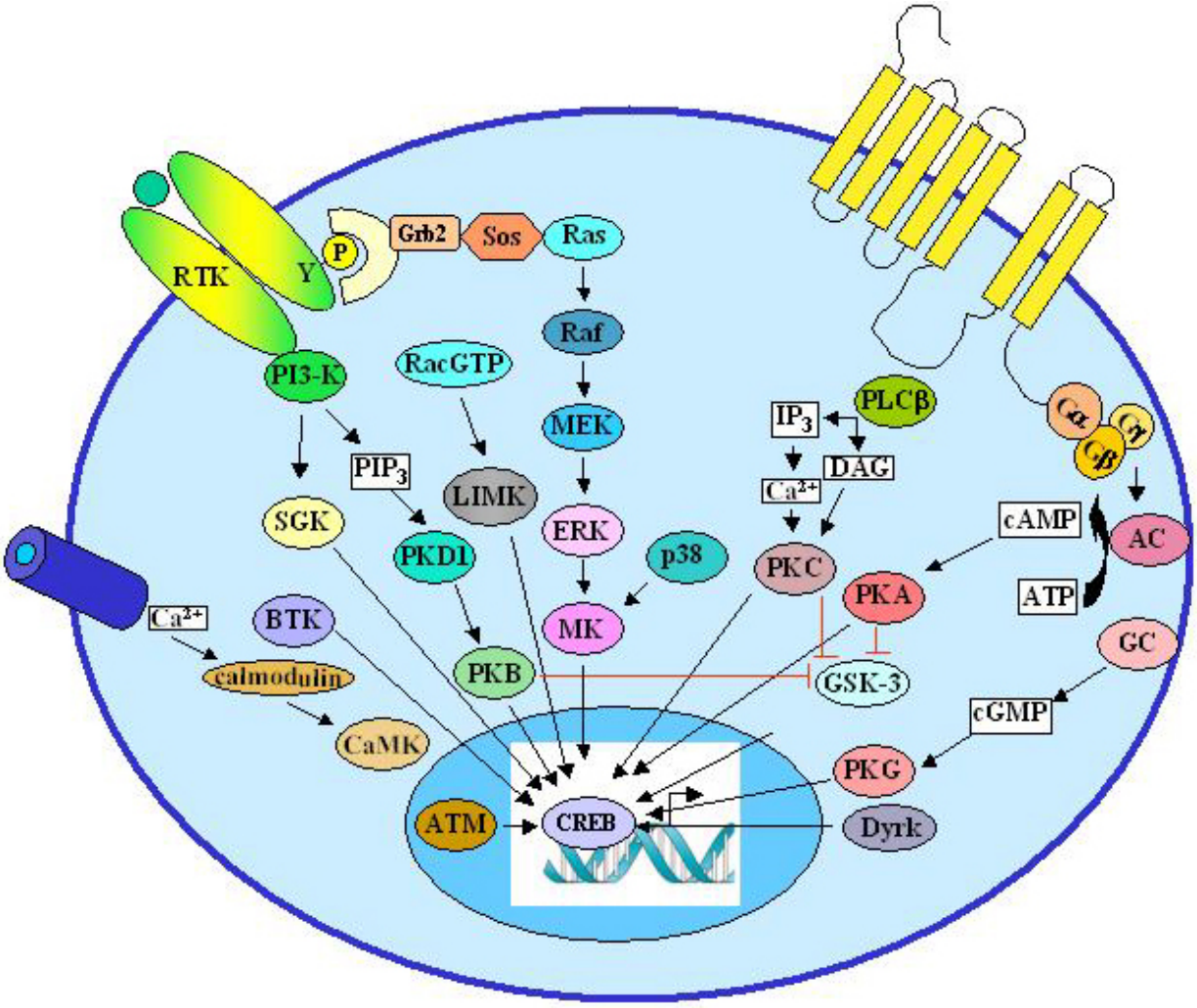

Figure 2. A plethora of stimuli, received by different receptors and transmitted through distinct pathways, converges to CREB. These pathways do not operate isolated, but can positively or negatively cross-talk with each other thereby amplifying and prolonging or inhibiting the signal. This interaction between different pathways can modify the amplitude and kinetics of CREB phosphorylation and hence affect the transcriptional activity of CREB. The red arrows indicate inhibition of a peculiar pathway. AC: adenylate cyclase, DAG: diacylglycerol, GC: guanylate cyclase, Grb: growth factor receptor-bound protein, IP3: inositol triphosphate , PI3K: phosphoinositide-3 kinase, PIP3: phosphatidylinositol (3,4,5)-triphosphate, PLC: phospholipase C, RTK: receptor tyrosine kinase, SOS: son of sevenless.

questions in the field of CREB phosphorylation research are "Why is CREB target of approximately 20 different protein kinases?" and "What is the impact of multiple phosphorylation events on CREB?" CREB mediates stimulus-dependent transcription of genes whose products are involved in various cellular processes. A plethora of stimuli, signaling through different pathways, induce phosphorylation-dependent activation of CREB (Figure 2). This stimulus-triggered phosphorylation of CREB is mediated by distinct protein kinases. The existence of numerous CREB kinases expands the diversity of signals to which CREB can respond. In addition, cross-talk between signaling pathways permits a specific signal to be transmitted by distinct signaling pathways converging to CREB, resulting in amplification and endurance of the signal. This may in turn affect the kinetics and stoichiometry of CREB phosphorylation and hence the transcriptional activity of CREB (9). We have previously shown that forskolin-activated PKA can regulate the activity of MAP kinase p38, which subsequently activates MSK1. MAP kinase p38-mediated phosphorylation of CREB is postponed compared to PKA-triggered CREB phosphorylation. In fact, at time points when PKA is in the refractory phase, p38 becomes activated. Consequently, the original stimulatory effect of PKA is transferred to p38, assuring prolongation of the phosphoCREB kinetics (112). Similarly, the CaMK and MAPK/RSK signaling pathways may modulate the kinetics of depolarization-induced CREB phosphorylation in hippocampal neurons (113). The existence of multiple CREB kinases offers the possibility for cell-specific gene expression of CREB target genes, as cell-specific expression of particular CREB kinases can

contribute to cell-specific phosphorylation of CREB. Different phosphorylation events may fine-tune the transcriptional activity of CREB. Depending on the sites phosphorylated by CREB kinases, CREB may be converted into a transcriptional active or inactive transcription factor. Sequential phosphorylation events may further add to the accurate regulation of CREB's transcriptional properties.

6. PERSPECTIVE

Although several studies have shown that single phosphorylation of CREB at serine-133 is necessary for CREB activation, it may not be sufficient to fully activate CREB in response to specific stimuli. Additional phosphorylation events besides serine-133 have been described, but the biological consequences of these phosphorylation events have not been fully explored. It can be imagined that these modifications may affect the interaction with other CREB-interacting proteins besides CBP/p300. Alternative, non-serine-133 phosphorylations may trigger other post-translational modification events such as ubiquitination and subsequent proteasom-mediated degradation. It is also possible that there is a hierarchy in the phosphorylation events. Multiple phosphorylations to activate CREB was illustratively compared with opening of a briefcase lock: "A scheme in which different combinations of phosphorylations on CREB and its coactivators (present at any one time) control gene expression resembles the working of a briefcase lock, in which each wheel must be in the correct position simultaneously for the lock to disengage. But, dynamic scenarios also present themselves, more like a traditional combination lock on a safe, in which different wheel positions are required sequentially." (114). The multiple phosphorylation events may allow a graded regulation of CREB activity, thus offering an additional level of transcriptional control of CREB target genes. Recent studies with Ets-1 revealed that the binding activity of this transcription factor is inhibited in direct proportion to the number of sites phosphorylated within the serine rich region of the protein (115). The biological effect of multisite phosphorylation on CREB activity forms an important challenge to elucidate the molecular mechanisms of CREB-mediated transcription. The generation of phosphospecific antibodies directed against other phosphoacceptor sites in CREB may offer a helpful tool in establishing a functional role of these phosphorylation events in response to different stimuli. Moreover, transgenic animal models expressing CREB mutants with multiple substitutions of phosphoacceptor sites may expand our knowledge on the biological functions of this transcription factor. Another puzzling observation is that phosphorylation at a specific site increases CREB activity in one cell-type, but represses it in another cell. Thus, despite 20 years of intensive CREB research, this protein remains enigmatic in many aspects.

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Abbreviations: ATM: Ataxia-telangiectasia mutated, ATR: ATM-Rad3-related kinase, bFIK: bFGF-inducible kinase, BTK: Bruton's tyrosine kinase, CaMK: Ca^{2+}/calmodulin kinase, CKII: casein kinase 2 or protein kinase CK2, GSK3: Glycogen synthase kinase-3, HIP: hypoxia-induced kinase, LIMK1: LIM kinase 1; MK: Mitogen-activated protein kinase activated protein kinases, PKA: cAMP-dependent protein kinase, PKB: protein kinase B, PKC: protein kinase C, PKG: cGMP-dependent protein kinase, SGK: serum/glucocorticoid-inducible kinase, TSSK5: testis-specific serine/threonine kinase 5.

Key Words: ATM, casein kinase II, Testis-Specific Serine, Threonine Kinase 5, LIM kinase 1, DYRK, MAPKAPK, PKA, PKB, PKC, PKG, Ca^{2+}, Calmodulin-Dependent Kinases, Glycogen Synthase Kinase-3, Hypoxia-Induced Kinase, Bruton's Tyrosine Kinase, bFGF-inducible kinase, serum/glucocorticoid-inducible kinase, Review

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