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Dynamic Multiphosphorylation Passwords for Activity-Dependent Gene Expression

Minireview

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Synapse-to-nucleus signaling leading to CREB-mediated transcription is important for neuronal plasticity. Nuclear CREB phosphorylation at Ser133 allows convergence of multiple kinase pathways driven by neuronal activity and links them to transcriptional activation. But, can various pathways share a common effector mechanism (phosphorylating Ser133) while generating distinct patterns of gene expression? We review three *Neuron* articles that highlight novel ways Ca^{2+} signals can trigger multiple phosphorylation events working in combination to control CREB and its interaction with coactivator molecules.

Information processing of signals at the nucleus is critical for plasticity in the brain. At the heart of this processing are signaling pathways between electrical and chemical signals at the cell surface and a plethora of transcription factors in the nucleus. CREB, the best characterized member of a family of basic DNA binding-leucine zipper (b-ZIP) transcription factors, has accumulated an impressive résumé of roles in brain plasticity in a wide range of animal species. Behavioral genetic studies have demonstrated its key role in memory consolidation, as well as other interesting phenomena, such as circadian rhythmicity and addiction. CREB is well positioned to participate in intricate nuclear computations, with responsiveness to multiple intracellular cascades involving CaM kinases, cAMP/PKA, and MAP kinases and to multiple external signals, including neurotransmitters and growth factors capable of recruiting the intracellular players (reviewed in West et al., 2001). In addition to combinatorial processing, nuclear processing of synapse-to-nucleus signals can occur with considerable temporal sophistication (Mermelstein et al., 2001).

According to the prevailing view, Ser133 of CREB represents a convergence point for phosphorylation by multiple kinases and is necessary for a molecular switch that controls gene expression. For example, in hippocampus and cortex (the brain areas most relevant for memory consolidation) behavioral or depolarizing/synaptic stimuli acting through L type Ca^{2+} channels and NMDA receptors recruit a fast nuclear calmodulin/CaM kinase IV-mediated pathway to CREB Ser133 (reviewed in West et al., 2001; Bito et al., 1996; Deisseroth et al., 1998; Ho et al., 2000; Kang et al., 2001; Ribar et al., 2000), followed after strong stimuli by a more slowly developing but long-lasting Ras/MAP kinase/Rsk-mediated pathway (Wu et al., 2001; reviewed in West et al., 2001). Studies of Ser133 phosphorylation have been advanced by the availability of site-selective, phospho-specific antibodies (Ginty et al., 1993).

However, simple Ser133 phosphorylation is necessary, but not sufficient, to achieve transcription in activated neurons (Bito et al., 1996). Many groups have noted that stimuli capable of inducing CRE-dependent gene expression are only those that are able to induce particularly long-lasting Ser133 phosphorylation, suggesting the importance of the time integral of phospho-CREB. Possible additional regulatory factors include the adaptors linking CREB to the basal transcription complex, including the potential coactivators CREB binding protein (CBP) and p300. The importance of CBP in PKA-dependent CREB function has been demonstrated by several groups, and it is important to note that humans with a mutated CBP allele suffer from mental retardation, among other abnormalities (Rubinstein-Taybi Syndrome).

With increasingly close scrutiny of CBP, it has become clear that CBP can itself be actively regulated and could represent more than a mere passive link to the transcriptional machinery (Hu et al., 1999; Hardingham et al., 1999), though the relevance of these findings to neuronal activity-dependent CREB function per se is not clear. Simply recruiting CBP itself to CREB can promote increases in transcription (Cardinaux et al., 2000; for discussion see Mayr and Montminy 2001). An important paper from Ghosh and colleagues characterized a candidate "coactivation" event: phosphorylation of CBP itself (Hu et al., 1999). Hu et al. (1999) found a Ca^{2+}-responsive transactivation domain in the N-terminal region of CBP, between aa 227 and aa 460, along with a distinct C-terminal domain. Glutamate induction of CBP N-terminal-mediated transcription was inhibited by KN-62, implicating a CaM kinase, but not by pharmacological inhibitors of MEK and PKA. Expression of constitutively active forms of either CaM kinase II or CaM kinase IV was sufficient to induce transactivation via both the N-terminal and the C-terminal domains of CBP. However, it should be pointed out that CaM kinase activation of CBP does not in itself prove a role for CBP in activity-dependent CREB function. Though several groups have found that the CBP/p300 inhibitor E1A can interfere with CREB-dependent gene expression, proof of CBP's involvement in this situation has been lacking.

Knowledge about the mechanism and precise locus of Ca^{2+} regulation of CBP has also been limited up to now. In this issue of *Neuron*, Impey et al. (2002) have carried out a painstaking analysis to identify CBP Ser301 as a target of regulation. Using an anti-phospho-Ser301 antiserum, they were able to demonstrate a 2.5-fold increase in p301-CBP within 2 min after stimulation with NMDA. Mutation of Ser301 to alanine prevented CaMKIV phosphorylation in vitro, and block of CaMK activity with KN93 attenuated the ability of NMDA to generate Ser301 phosphorylation in neurons. Therefore, rapid generation of Ser301 phospho-CBP is a role of CaMKIV, along with rapid generation of Ser133 phospho-CREB.

The involvement of CaMKIV in activity-regulated CREB-dependent transcription is widely accepted. Could phosphorylation of CBP-Ser301 suffice to explain the involve-

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ment of CaMKIV in CREB function, since other signaling pathways might fulfill the requirement for CREB Ser133 phosphorylation? Many findings from Impey et al. (2002) and others instead demonstrate the opposite: that CaMKIV-CBPSer301 activation is not necessary or sufficient to explain CaMKIV activation of CREB-dependent transcription (or indeed activation of CBP itself). CaMKIV-CBPSer301 activation is not necessary, since, as Impey et al. (2002) and others (Cardinaux et al., 2000) have shown, simply stabilizing the CREB-CBP complex with the CREB mutant CREB-DIEDML, even in the presence of dominant-negative CaMKIV, promotes CRE-dependent transcription (though further enhancement is possible). CaMKIV-CBPSer301 activation is likely not sufficient, since even if CBP is involved in activity-dependent CREB function, genetic blockade of CaMKIV markedly inhibits CREB phosphorylation on the critical Ser133 in vitro and in vivo, over a broad range of time points, as discussed above (Bito et al., 1996; Ho et al., 2000; Kang et al., 2001). Finally, constitutively active CaMKIV appeared to markedly stimulate GalCBP S301A transcription (Impey et al., 2002), demonstrating that CBP Ser301 phosphorylation does not fully explain the CaMKIV activation on CBP.

Clearly some nontrivial questions remain. It will be necessary to look for additional CaMKIV phosphorylation sites on CBP, particularly in the C-terminal region. A role for CAMKII was not excluded. Nevertheless, the findings of Impey et al. (2002) represent an important advance in specifically identifying a site of CaMKIV action potentiating CBP-dependent transcription, one that does not appear to be accessible to PKA or MEK/MAPK signaling, unlike CREB Ser133 phosphorylation.

In two additional articles in this issue of *Neuron*, Kornhauser et al. (2002) and Gau et al. (2002) have provided intriguing new evidence regarding an old hypothesis, that signaling via CREB Ser133 might be supplemented by other phosphorylation events on CREB itself. Back in 1989, Montminy and colleagues pointed out that the primary sequence of CREB contained "a cluster of protein kinase A, protein kinase C, and casein kinase II consensus recognition sites," commenting that "proximity of these potential phosphorylation sites to one another indicates that they may interact either positively or negatively to regulate CREB bioactivity" (Gonzalez et al., 1989). Maurer and colleagues (1994) found that phosphorylation of Ser142 by CaMKII blocks CaMKIV activation of CREB and that mutation of Ser142 to alanine enhanced the ability of Ca^{2+} influx to activate CREB (Sun et al., 1994). A molecular mechanism for this effect became clear with structural studies, revealing that Ser142 phosphorylation will block the interaction of CREB's kinase-inducible domain (KID) with the KIX domain of CBP (Parker et al., 1998). So, the impact of Ser142 seemed settled as a form of inactivation of excitation-transciption coupling. However, the studies of Gau et al. (2002) and Kornhauser et al. (2002) raise the possibility that Ser142 phosphorylation can participate positively in CREB-mediated transcriptional activation.

Gau et al. (2002) studied the functional significance of S142 in the context of circadian rhythm, by use of their own CREB-Ser142 antibody and by generation of a knockin mouse with CREB Ser142 mutated to alanine. They found that phosphorylation of Ser142 parallels that of Ser133 in a number of important respects. At both sites, the phosphorylation oscillated with a circadian rhythm, with a peak at subjective dawn and a valley at subjective dusk; the phosphorylation responded to light 2 hr after, but not 2 hr before, subjective dusk, and the phosphorylation responded to glutamate application in SCN slices at the phase in the cycle when light sensitivity had been observed. Apparently, phosphorylation of Ser142, like that of Ser133, participates in the circadian oscillator. This was tested further by examining resetting of the circadian oscillator, either produced by light in subjective early night, causing a phase delay in nocturnal activity, or produced by light just before dawn, causing a subsequent phase advance in nocturnal activity. In both cases, the absolute amount of phase shift was significantly attenuated by the Ser142A modification. The mutation caused a concomitant block of the induction of c-Fos protein and *mPer1*, but not phosphorylation of CREB at Ser133 or induction of *mPer2*.

There are several aspects to the unique value of this elegant paper. The findings on CREB Ser142 phosphorylation and its stimulatory role in gene expression result from in vivo activity patterns affecting endogenous genes. Any model of activity-dependent CREB function must, therefore, reckon with the conclusion that Ser142 phosphorylation can stimulate expression of a CREB-dependent gene. The clear behavioral consequences of interfering with Ser142 phosphorylation further demonstrate that activation of this residue is crucial to a high-level function of the intact brain.

In vivo Ser142 phosphorylation appeared to be lower in pyramidal neurons of the hippocampus than in the SCN, suggesting that phospho-Ser142 may not occur robustly in all cell types, but how can these findings be reconciled with the view that Ser142 phosphorylation is inhibitory? Here's where the experiments from Michael Greenberg's group make a strategic entry. Kornhauser et al. (2002) developed an impressive set of antibodies specific for CREB phosphorylated at Ser133, Ser142, or Ser142 and Ser143. Using the antibodies to study cortical neurons, they showed that stimulation of cAMP/PKA or neurotrophin signaling pathways by forskolin or BDNF *only* generated phospho-Ser133. Furthermore, either Ser143A mutation or Ser142 and Ser143 double mutation spared resulting CREB-dependent gene expression. On the other hand, K^+-depolarization-induced Ca^{2+} entry not only caused the well-known Ser133 phosphorylation, but also produced Ser142 and Ser143 phosphorylation. This raised the possibility that Ca^{2+} signaling might enjoy a "private line" to a specific form of phospho-CREB that might be recognized specifically by downstream mechanisms. Indeed, kinases known to be synaptically activated seem to be able to phosphorylate these sites (CaMKII on Ser142, CKII on Ser143 or Ser142).

But as Kornhauser et al. (2002) showed next, this private line comes with more responsibility than privilege. PKA signaling works in blissful unawareness of the Ser142/Ser143 status, while Ca^{2+} signaling carries not only the ability to phosphorylate these residues, but also a requirement to phosphorylate them in order to get maximal CRE-dependent gene expression. The combined mutation of Ser142 and Ser143 (or of Ser143 alone) removes about half of the Ca^{2+}-dependent Gal4CREB

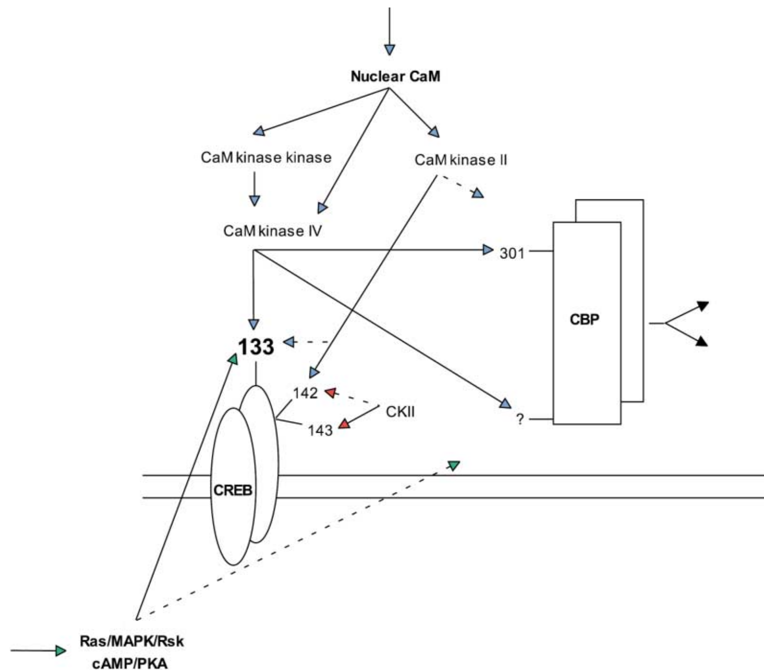

Figure 1. Multiphosphorylation Tuning of CREB-Dependent Gene Expression

Several pathways converging on the nucleus can access the required Ser133 on CREB, but the Ras/MAPK/Rsk and PKA pathways do not seem to access the modulatory CREB Ser142/Ser143 or CBP Ser301. Elevations in nuclear Ca^{2+}/CaM, on the other hand, are able to recruit CREB Ser133, CREB Ser142 (which may then allow Ser143 activation by CKII), CBP Ser301, and likely, other residues, through an array of nuclear CaM kinases. CBP is certain to play additional roles, but a role in activity-dependent CREB function remains unclear and may depend on the CREB Ser142/Ser143 status. Diversity in nuclear expression levels of these kinases and differential recruitment of different signaling pathways, depending on the synaptic stimulus, could lead to markedly different patterns of gene expression with CREB Ser133 phosphorylation as the common theme.

activation above baseline. (An interesting wrinkle is that under conditions where CREB dimerization is important, mutation of Ser142 alone actually enhances transcription; this is likely relevant to the earlier work on inhibitory effects of phospho-Ser142, but it is unclear to what extent which effect would predominate on a physiological promoter with a more complicated transcription factor assembly and chromatin structure). At present, it is not known what fraction of CREB molecules in a cell will experience all of these additional phosphorylations.

Despite the presumed stimulatory role of the dual Ser142/Ser143 phosphorylation (since the two alanine mutations inhibit transcription), the combined phosphorylation of Ser142 and Ser143 in vitro appears to virtually abolish CREB interaction with the KIX domain of CBP. It is interesting to speculate that PKA uses CREB Ser133 and CBP according to the canonical theory, but depolarization activates a separate inhibitory pathway to CREB that must be relieved by Ser142/Ser143 phosphorylation; for example, an inhibitory protein binding to the KID of CREB that is kicked off by the dual phosphorylation. Though speculation on the identity of this factor could be far-ranging, one intriguing possibility is that the inhibitor is CBP itself—stuck firmly to phospho-Ser133 CREB when Ser142/Ser143 are unphosphorylated, but inactive due to a twist of the transcription factor complex after depolarization that we don't fully understand. Indeed, this misbound CBP would be dominant-negative until it gets removed or repositioned by Ser142/Ser143 phosphorylation, thereby allowing another activator like p300 to bind or another domain of CBP to be called into action. Competition between functional coactivators and inactivating coactivator-like components or between different transcription factors competing for the same coactivator clearly provide additional opportunities for inhibitory, as well as stimulatory, influences of the co-"activators."

On the face of it, Kornhauser et al. (2002) cast doubt on the stimulatory role of CBP binding itself, as do the results of Gau et al. (2002). Could CBP, despite its well-established role in PKA-dependent CREB function and its own activity-dependence, never even bind to CREB during activity-dependent CREB transcription? Other coactivators could do the job (E1A inhibition of activity-dependent CREB function, as found by some groups, implicates either CBP or the related coactivator p300). However, Impey et al. (2002) provide support for physical proximity of CBP itself using a chromatin immunoprecipitation experiment. Increased amounts of CRE promoter regions are found to be rapidly associated with CBP after NMDA stimulation, apparently no less than with PKA stimulation. Perhaps it is another region of CBP, not the KIX domain, which is bound to the CREB complexes after depolarization, or perhaps CBP binds to another nearby factor, though a stimulatory role for such CBP binding remains unproven. Experiments assessing physical association between CREB and CBP using a FRET strategy in neurons (Mayr and Montminy, 2001) may be helpful in resolving under what conditions CBP actually associates with CREB.

Kinetic considerations loom large. Phospho-Ser142 and phospho-Ser143 not only develop more slowly than phospho-Ser133, but also decay more quickly (Kornhauser et al., 2002). One can then begin to break down the Ca^{2+} activation process into temporal stages. Given that CaMKIV phosphorylates Ser133 quickly and phospho-Ser133 CREB will readily interact with CBP, Ca^{2+} signals might be expected to immediately achieve the same functional phospho-Ser133 activation state produced by PKA recruitment. Given that the P142/P143 state is transient, a purely phospho-Ser133 state may appear again after a brief ($\sim$30 min) delay. The intervening Ser142/Ser143 phosphorylation state could involve the recruitment of a non-CBP pathway. Could this temporal scenario explain why duration of CREB Ser133 phosphorylation is so important—to ensure maximal

P133 potency when full Ser142/Ser143 recruitment is achieved or, perhaps, in other scenarios, to outlast the Ser142/Ser143 inhibition of CBP binding? Which of these stages is the active one for a particular gene may depend on which coactivators are present and which signaling pathways have activated them.

Some dissonance might be expected at this stage of discovery, given that experimental differences abound among these three important papers, perhaps with interesting consequences. For example, Kornhauser et al. (2002) used cortical neurons exposed to potassium depolarization in 9.2 mM Ca^{2+}, Impey et al. (2002) focused on NMDA stimulation of hippocampal neurons, and Gau et al. (2002) used SCN neurons with chiefly behavioral stimuli. Are cell type differences relevant? In the SCN, CaMKIV expression may be relatively low (Nakamura et al., 1995), so perhaps CBP is deprived of its Ser301 and other phosphorylations and, indeed, acts as a dominant-negative, sitting on Ser133 CREB and blocking (for example) p300 association, unless knocked off by CaMKII-regulated Ser142 phosphorylation. In the hippocampus, CaMKIV is abundant, so perhaps CBP simply cannot act in this dominant-negative fashion. Strategies to test this scenario are clear enough: it will be critical to determine how p300 associates with CREB under the various phosphorylation conditions in different cell types and to clarify activity dependence of p300 or other elements of the initiation complex. No doubt additional activity-dependent covalent modification of the coactivators, other transcriptional machinery components, or chromatin itself will be identified along the way, and several groups have already begun to identify such additional targets. In this context it is important to point out the difference between quantitative and qualitative effects on CREB-dependent gene expression. As shown repeatedly by many groups and again here by Kornhauser et al. (2002), the CREB Ser133 requirement is virtually absolute, regardless of the stimulation pathway, while the other phosphorylations play important but more graded roles in tuning transcriptional responses (see Figure 1).

A scheme in which different combinations of phosphorylations on CREB and its coactivators (present at any one time) control gene expression resembles the workings 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. Either way, these new papers, along with earlier work, suggest that the signaling requirements to achieve CRE-mediated gene expression are more complex than those of a simple on-off switch. The timing and the context of incoming information are critical in determining whether CREB succeeds in driving gene expression.

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