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Opposing effects of Elk-1 multisite phosphorylation shape its response to ERK activation

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Multisite phosphorylation regulates many transcription factors, including the serum response factor partner Elk-1. Phosphorylation of the transcriptional activation domain (TAD) of Elk-1 by the protein kinase ERK at multiple sites potentiates recruitment of the Mediator transcriptional coactivator complex and transcriptional activation, but the roles of individual phosphorylation events had remained unclear. Using time-resolved nuclear magnetic resonance spectroscopy, we found that ERK2 phosphorylation proceeds at markedly different rates at eight TAD sites in vitro, which we classified as fast, intermediate, and slow. Mutagenesis experiments showed that phosphorylation of fast and intermediate sites promoted Mediator interaction and transcriptional activation, whereas modification of slow sites counteracted both functions, thereby limiting Elk-1 output. Progressive Elk-1 phosphorylation thus ensures a self-limiting response to ERK activation, which occurs independently of antagonizing phosphatase activity.

Multisite protein phosphorylation increases the complexity of functional signaling outputs that can be generated from single protein kinase inputs. It can set thresholds for activity or transform graded signals into switch-like responses (1–4). Many transcription factors and their interacting regulatory proteins are subject to multisite phosphorylation, which allows distinct aspects of protein function—including protein turnover, nuclear import and export, and specific protein interactions—to be controlled independently (5). However, in general, the dynamics and functional roles of individual phosphorylation events are incompletely understood.

The ternary complex factor (TCF) subfamily of Ets-domain transcription factors, consisting of Elk-1, SAP-1, and Net, provides an example of multisite phosphorylation in transcriptional activation. TCFs, together with their partner protein SRF, function in many biological processes by coupling SRF target genes to mitogen-activated protein kinase (MAP kinase) signaling (5). Mitogenic and stress stimuli induce phosphorylation of TCF C-terminal transcriptional activation domains (TADs) at multiple S/T-P (Ser or Thr-Pro) phosphorylation sequences, of which eight are conserved across the family (Fig. 1A and fig. S1) (6–11). Two MAP kinase docking sites, the D-box and the Phe-Gln-Phe-Pro (FQFP) motif, control phosphorylation of these sites (12–15). Multisite phosphorylation triggers transcriptional activation by TCFs, facilitating their interaction with the Mediator transcriptional coactivator complex (16–19), but the kinetics with which the different sites are phosphorylated, and whether they serve distinct functions, remain unclear.

To obtain atomic-resolution insights into phosphorylation of the Elk-1 TAD, we used nuclear magnetic resonance (NMR) spectroscopy (20) to monitor its modification by recombinant ERK2 in vitro (Fig. 1B and fig. S2A). Time-resolved NMR experiments revealed that each phosphorylation proceeded efficiently but at markedly different rates. Phosphorylation of Thr399 and Ser384, which flank the central Phe-Trp (FW) motif implicated in Mediator interaction (18), occurred faster than

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Fig. 1. Multisite phosphorylation of Elk-1 TAD. (A) Linear outline of Elk-1 fast (red), intermediate (blue), and slow (yellow) S/T-P phosphorylation sites. Kinase docking motifs are shown in green; the FW residues (purple) are essential for Mediator association. (B) NMR analysis of Elk-1 TAD (amino acids 309 to 429) phosphorylation with recombinant ERK2. Left: 2D $^1\text{H}$-$^1\text{N}$ NMR spectrum of unphosphorylated Elk-1 (black), with phosphorylation site signals color-coded as in (A). Right: Overlay of 2D NMR spectra of phosphorylated [gray and color-coded as in (A)] and unmodified Elk-1 (black). (C) Time-resolved modification curves of individual Elk-1 sites upon phosphorylation with ERK2; error bars denote differences between replicate experiments on two independent samples. (D) Time-course Western blot of GST–Elk-1 TAD phosphorylation and phosphorylation site–specific antibody detection.
modification of Thr369, Thr418, and Ser423, whereas residues Thr337, Ser437, and Thr337 were modified more slowly (Fig. 1C), which we confirmed by immunoblotting (Fig. 1D). Chemical shift analysis (C, C) showed no stable secondary structure elements in unmodified or phosphorylated Elk-1 TAD (fig. S2B).

As a first step toward understanding the basis for the phosphorylation sites’ differential kinetic behavior, we devised a reaction model based on Michaelis-Menten enzyme kinetics. To simplify the mathematical treatment, we grouped Elk-1 sites into three classes: fast (Thr369 and Ser384), intermediate (Thr369, Thr418, and Ser390), and slow (Thr337, Thr423, and Ser423). We assumed that ERK2 phosphorylation is distributive, that enzymatic rate constants ($k_{on}$) are similar for all sites, and that the different sites have relative affinities for ERK2 modeled by increasing Michaelis-Menten constants ($K_{m,fast} < K_{m,int} < K_{m,slow}$) (Fig. 2A and fig. S3A) (2D). This model, which recapitulated the measured kinetics of in vitro Elk-1 phosphorylation well (Fig. 2B), predicted that removal of fast or intermediate sites should increase the phosphorylation rates of other sites. To test this idea, we analyzed the phosphorylation kinetics of Elk-1 TAD mutants in which we substituted all fast or intermediate phosphoacceptor residues with alanines (Elk-1F: Thr369 → Ala, Ser384 → Ala; Elk-1I: Thr354 → Ala, Thr364 → Ala, Ser390 → Ala) (Fig. 2A). In the fast-site mutant Elk-1F, phosphorylation rates of intermediate and slow sites increased, whereas those of the fast and slow sites increased in the intermediate-site mutant Elk-1I; in both cases, the altered kinetics fit well with those predicted by the model (Fig. 2B and fig. S3B). Thus, even the fast sites are not phosphorylated at the maximum possible rate in the wild-type protein. Moreover, phosphorylation of an Elk-1 TAD mutant in which Thr369 and Ser390 were replaced with aspartates was similar to Elk-1F, excluding the possibility that fast-site phosphorylation primes later modification events (fig. S3B).

To gain more insight into the factors affecting individual sites’ phosphorylation kinetics, we assessed the role played by primary sequence. To do this, we exchanged the sequences surrounding the fast Thr369 and slow Ser423 sites. This also effectively changed their reactivities, which suggests that these sites’ phosphorylation rates reflect their position relative to ERK docking sequences, rather than intrinsic differences in reactivity (Fig. 2C). We therefore examined the contributions of the D-box and FQFP ERK docking motifs to each site’s phosphorylation kinetics. Deletion of the D-box decreased the rates of Thr369, Thr418, and Thr369 phosphorylation but increased the rates of Ser384, Thr423, and Ser423 modification (Fig. 2D). In contrast, deletion of the FQFP motif decreased the rate of Ser423 phosphorylation but enhanced modification of intermediate sites, including adjacent Ser390, with no effect on the C-terminal sites (Fig. 2D). Thus, the ERK docking motifs differentially affect each phosphorylation site’s competitive behavior. Previous studies showed that Elk-1 TAD phosphorylation by JNK and p38 MAP kinases differs from phosphorylation by ERK (10, 21–24) and that this reflects differences in their docking interactions (12, 14, 15, 25). Indeed, these kinases exhibited site preferences and phosphorylation rates that were distinct from that of ERK2 (fig. S3C). Taken together,
Elk-1 target genes (Fig. 3A). Alanine substitutions at Elk-1 enhanced proliferation, and Mediator binding. (A) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of TCF target gene transcription in reconstituted TKO MEFs. Cells were reconstituted with wild-type mouse Elk-1 (WT) or fast-site (F), intermediate-site (I), slow-site (S), fast- and intermediate-site (FI), or alanine-substituted FW motif (FW) mutants. (B) Effects of individual slow-site alanine substitutions on Egr1 expression. Data are expressed relative to expression of GAPDH. In (A) and (B), cells were stimulated with TPA (50 ng/ml) and/or medium.

To test whether the different kinetic classes of Elk-1 TAD phosphorylation sites are functionally equivalent, we expressed Elk-1 mutants in fibroblasts derived from TCF-deficient (Elk1−/−; Elk3−/−; Elk4−/−) triple-knockout mouse embryos (TKO MEFs; fig. S4, A to C). In these cells, immediate-early (IE) gene expression is defective, but expression of wild-type mouse Elk-1 restored the IE transcriptional activation seen in wild-type MEFs after activation of ERK by treatment with TPA (12-O-tetradecanoylphorbol-13-acetate) (fig. S4D). As expected, alanine substitutions of fast and/or intermediate sites, or of the FW motif, greatly diminished or abolished the ability of Elk-1 to activate TCF-SRF target gene transcription after TPA stimulation (Fig. 3A). Surprisingly, however, mutation of the slow sites substantially enhanced Elk-1–mediated activation of TCF-SRF target genes (Fig. 3A). Alanine substitutions at individual slow sites also increased Elk-1 activity, with Thr418 exhibiting the greatest effect (Fig. 3B and fig. S4, E to G). TCF-SRF signaling is important for cellular proliferation (26, 27), and TKO MEFs proliferated more slowly than wild-type MEFs. The reconstituted TKO MEFs exhibited enhanced proliferation rates, which correlated with the ability of each mutant to promote transcriptional activation (Fig. 3C).

Phosphorylation of Elk-1 promotes transcriptional activation by facilitating its MED23-dependent interaction with the Mediator complex (16–18). We therefore investigated whether the different transcriptional activities of the Elk-1 mutants reflected alterations in Mediator binding. We prepared extracts of TKO cells expressing wild-type or mutant Elk-1 proteins and assessed Elk-1 association with Mediator by coimmunoprecipitation of the MED23, MED24, and MED16 subunits. Consistent with the transcription experiments, Elk-1–Mediator interaction was induced by TPA stimulation and dependent on the FW motif; it was abolished by alanine substitutions of fast and intermediate sites, and increased in the slow-site Elk-1 mutant (Fig. 3D). We obtained similar results when we used glutathione S-transferase (GST)–Elk-1 TAD proteins to recover Mediator proteins from unstimulated NIH3T3 cell extracts (Fig. 3E). In this assay, ERK2 phosphorylation time-course experiments showed that Mediator recovery by the wild-type Elk-1 TAD was most efficient prior to modifications of the slow sites (fig. S5, A and B). Taken together, these data show that according to the sites involved, ERK2 phosphorylation promotes or inhibits transcriptional activation by Elk-1, which reflects alterations in Elk-1–Mediator interactions.

Next, we investigated Elk-1 TAD phosphorylation kinetics in vivo. Previous studies were unable to distinguish the progressive phosphorylation of fast and slow Elk-1 sites (6). However, by incubating cells at 25°C to slow down reactions, we confirmed that phosphorylation rates can be ranked in the order Ser384 > Thr364 > Thr418 and that different site classes exhibited a similar competitive behavior, as seen in vitro (fig. S6A). Reasoning that phosphorylation of the Elk-1 TAD might be sensitive to kinetic effects at limiting signal strengths, we titrated ERK activity using...
Fig. 4. Multisite phosphorylation of Elk-1 shapes the transcriptional response to ERK activation. (A) Kinetics of Elk-1 fast- and slow-site phosphorylation in cells treated with increasing concentrations of TPA. (B) Transcription rate of the TCF-SRF target gene Egr1 in TKO MEFs expressing wild-type Elk-1 or mutant Elk-1S. Precursor RNA was monitored by qRT-PCR after stimulation with different concentrations of TPA. Data are means ± SEM; n = 3. (C) Kinetics of Egr1 mRNA accumulation in cells as in (B) monitored by qRT-PCR. (D) Progressive Elk-1 phosphorylation by ERK has both activating (left and center) and inhibitory (right) effects on Mediator recruitment, as suggested by shading densities. A strong signal will rapidly reach the attenuated state shown at the right; a weak signal may reach this state only if sustained.

Increasing amounts of TPA. This both increased the maximal extent of ERK activation and advanced the time at which it occurred (fig. S6B). At low TPA concentrations, Elk-1 fast-site (Ser384) and slow-site (Thr418) modifications accumulated slowly over 1 hour, whereas at a saturating TPA dose they were maximal by 10 min. Both phosphorylations declined at late times, presumably owing to the action of Elk-1 phosphatases (Fig. 4A) (6, 25).

Having established that Elk-1 phosphorylation kinetics are tuned by signal strength, we investigated their relationship to transcriptional activation. We compared the ability of wild-type Elk-1 and the slow-site mutant Elk-1S to activate transcription in response to signals of differing strengths. At saturating TPA concentrations, both proteins activated Egr1 transcription with similar transient kinetics, although Elk-1S was much more active, reflecting the loss of the inhibitory sites (Fig. 4, B and C). At limiting TPA doses, however, their behaviors were markedly different. Whereas the activity of wild-type Elk-1 was almost maximal by 15 min, that of Elk-1S increased substantially beyond this time (Fig. 4B), resulting in prolonged Egr1 mRNA accumulation (Fig. 4C). Thus, progressive phosphorylation of the Elk-1 TAD by a single kinase, ERK, attenuates the transcriptional response of Elk-1, shaping it according to the strength and kinetics of ERK activation.

Our results show that phosphorylation of the Elk-1 TAD by ERK can either promote or inhibit Mediator interaction depending on the sites involved, thereby modulating transcriptional activation. Given that the TAD sequences are conserved in the other TCFs, our findings may also apply to them. The more rapidly phosphorylated sites are located in the substantially conserved central core of the TAD and are essential for transcriptional activation, lying close to the FW hydrophobic motif required for Elk-1-Mediator interaction (10, 18). Multisite phosphorylation of these residues might stabilize this interaction and perhaps also set a signaling threshold for it, similar to the way that multisite phosphorylation sets a threshold for the Sic1-Cdc4 interaction (29). In contrast, slowly phosphorylated sites located N- and C-terminal of the conserved TAD core act negatively. Their phosphorylation inhibits Mediator recruitment and limits transcriptional activation (Fig. 4D) and may also facilitate recruitment of negative regulators of Elk-1 activity. Together, these properties ensure that ERK phosphorylation of the Elk-1 TAD is self-limiting, whereby phosphorylation of slow sites attenuates TCF-SRF target gene expression under conditions of strong or sustained ERK signaling (Fig. 4D). Our results challenge the common assumption that multisite modification events act unidirectionally and can only be reversed or limited by antagonistic enzymes, such as phosphatases. Given the prevalence of such events in different biological processes, we expect that similar mechanisms may govern other regulatory interactions.

REFERENCES AND NOTES
VACCINES

Rapid development of a DNA vaccine for Zika virus

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Zika virus (ZIKV) was identified as a cause of congenital disease during the explosive outbreak in the Americas and Caribbean that began in 2015. Because of the ongoing fetal risk from endemic disease and travel-related exposures, a vaccine to prevent viremia in women of childbearing age and their partners is imperative. We found that vaccination with DNA expressing the premembrane and envelope proteins of ZIKV was immunogenic in mice and nonhuman primates, and protection against viremia after ZIKV challenge correlated with serum neutralizing activity. These data not only indicate that DNA vaccination could be a successful approach to protect against ZIKV infection, but also suggest a protective threshold of vaccine-induced neutralizing activity that prevents viremia after acute infection.


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SUPPLEMENTARY MATERIALS
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Editor's Summary

A function for multisite phosphorylation

Many transcription factors are regulated by phosphorylation on multiple residues. Mylona et al. analyzed multisite phosphorylation in the transcription factor Elk-1 and showed that it may protect against excessive activation (see the Perspective by Whitmarsh and Davis). Phosphorylation by the kinase ERK2 occurred at eight sites, but the sites were phosphorylated at different rates. Those that were phosphorylated more quickly promoted transcriptional activation. Those that were phosphorylated more slowly dampened excessive activation by ERK2s without needing a phosphatase or any other negative regulatory component.

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