

Multistep phosphorylation systems: tunable components of biological signaling circuits

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ABSTRACT Multisite phosphorylation of proteins is a powerful signal processing mechanism that plays crucial roles in cell division and differentiation as well as in disease. We recently demonstrated a novel phenomenon in cell cycle regulation by showing that cyclin-dependent kinase-dependent multisite phosphorylation of a crucial substrate is performed sequentially in the N-to-C terminal direction along the disordered protein. The process is controlled by key parameters, including the distance between phosphorylation sites, the distribution of serines and threonines in sites, and the position of docking motifs. According to our model, linear patterns of phosphorylation along disordered protein segments determine the signal-response function of a multisite phosphorylation switch. Here we discuss the general advantages and engineering principles of multisite phosphorylation networks as processors of kinase signals. We also address the idea of using the mechanistic logic of linear multisite phosphorylation networks to design circuits for synthetic biology applications.

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INTRODUCTION

Phosphorylation of proteins is the most abundant posttranslational modification used in regulatory mechanisms in eukaryotic cells (Khoury *et al.*, 2011). Advanced techniques of phosphoproteomics have led to the identification of tens of thousands of phosphorylated sites in protein kinase targets. A closer look at phosphoproteomes highlights one important feature: a large fraction of protein kinase targets contain multiple phosphorylation sites, and clusters of sites tend to be located within intrinsically disordered regions of the target proteins (Holt *et al.*, 2009; Tyanova *et al.*, 2013). Thus it appears that the mechanism of phosphorylation may hide yet another level of complexity arising from combinatorial patterns of multistep phosphorylation events. However, how this multisite phosphorylation code is to be read and transformed into meaningful signaling information remains unclear because the biochemical details of the signal processing logic of these multisite phosphorylation networks have not been studied until recently.

During multisite phosphorylation, phosphate groups are added in either a random or defined order to serine, threonine, or tyrosine

residues in kinase substrates. When a crucial set of key sites becomes phosphorylated, the downstream signaling switch will be triggered. Our recent studies shed light on a mechanism by which sequential multisite phosphorylation is used for processing of cyclin-dependent kinase 1 (Cdk1) signals in cell cycle regulation (Kõivomägi *et al.*, 2011a,b, 2013; McGrath *et al.*, 2013). Here we discuss the main engineering principles of molecular switches based on sequential multisite phosphorylation of Cdk1 targets. We also explore a range of possible but as-yet-unobserved properties that multisite phosphorylation networks may provide to the signal processing capacity of cellular systems in general.

MULTISITE PHOSPHORYLATION CONTROLS DESTRUCTION OF A CDK1 INHIBITOR

Cdk1-dependent phosphorylation events often lead to the generation of phosphorylated sequence motifs (phosphodegrons) that are recognized by the ubiquitination machinery and thereby marked for destruction (Hao *et al.*, 2007; Kõivomägi *et al.*, 2011a; Landry *et al.*, 2012). For example, phosphorylation-dependent destruction of a Cdk1 inhibitor protein called Sic1 helps to trigger S phase in budding yeast. Cdk1, when bound to G1- and S-phase cyclins, phosphorylates Sic1 in an ordered sequence at multiple sites, leading to the formation of phosphodegrons that are recognized by the ubiquitin ligase SCF-Cdc4. The sequential phosphorylation of Sic1 and other substrates depends on three important interactions between Cdk1 complexes and the disordered substrate chain (Figure 1, A and B): the active site of Cdk1 interacts with the consensus phosphorylation site, typically S/T-P or S/T-PxK/R (Khoury *et al.*, 2011);

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Abbreviations used: CDK, cyclin-dependent kinase; MAP kinase, mitogen-activated protein kinase.

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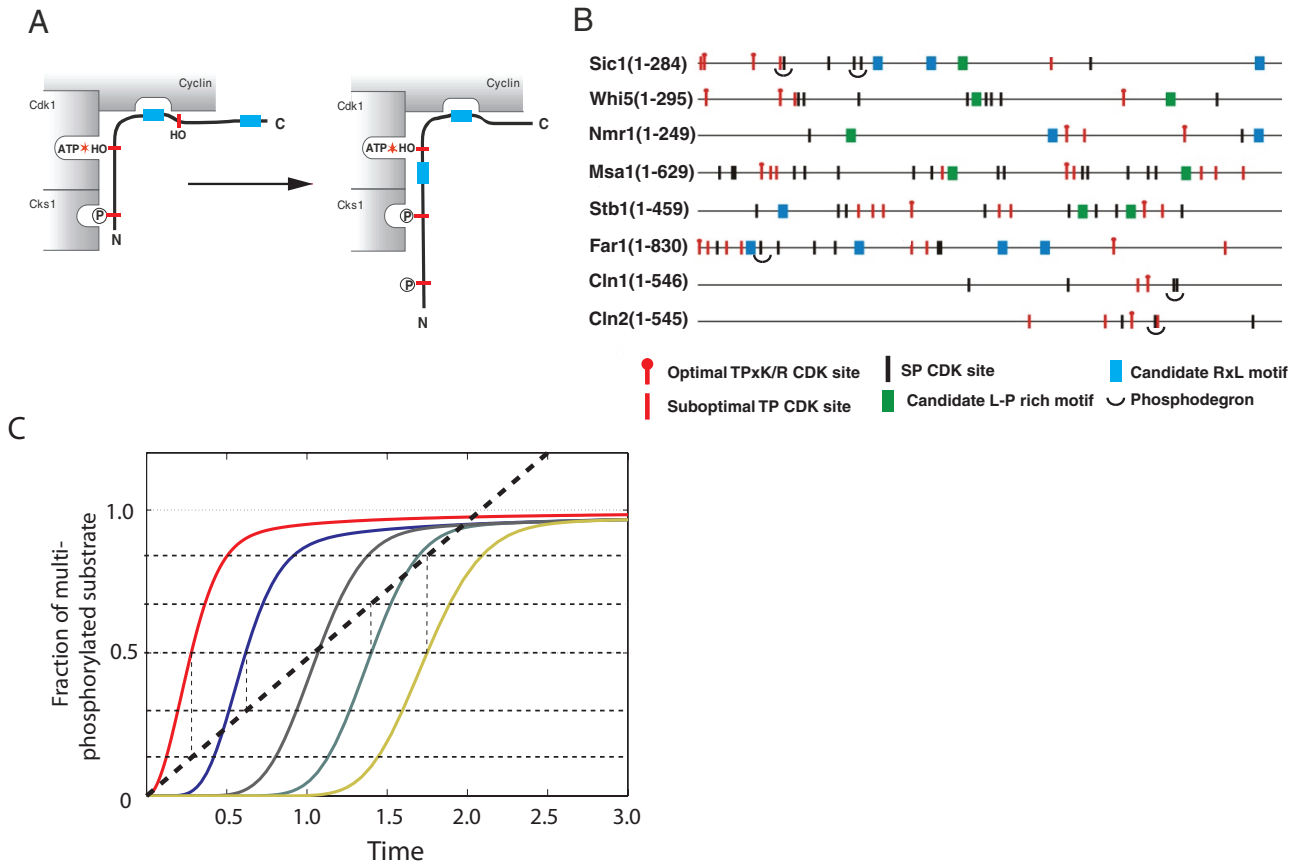


FIGURE 1: Multisite phosphorylation of Cdk1 targets. (A) A schematic model of a cyclin-Cdk1-Cks1 complex containing the catalytic Cdk1 subunit, the positive regulatory subunit (cyclin), and an accessory phosphate-binding subunit (Cks1). A disordered substrate is shown together with the docking motifs that interact with each of the subunits. These are as follows: the phosphorylated consensus site (S/T-P) interacts with the Cdk1 active site (red marks with -OH), a cyclin docking motif interacts with a hydrophobic pocket on the cyclin (blue boxes), and a phosphorylated threonine on the substrate interacts with Cks1 (red marks with -P). (B) Examples of multisite phosphorylation networks in selected Cdk1 targets of *Saccharomyces cerevisiae*. There are at least two types of cyclin-docking motifs in Cdk1 substrates. L-P rich motifs are specific docking sites for the G1-specific Cln2-Cdk1 complex (Bhaduri and Pryciak, 2011; Koivomagi *et al.*, 2011b). RXL motifs are docking motifs specific for the S-phase complex Clb5-Cdk1. Phosphodegrons are sequence motifs composed of two phosphorylated sites positioned 2–3 amino acids apart and recognized by the ubiquitin ligase SCF-Cdc4. Networks are mostly located in disordered regions of the proteins. (C) Graph depicting the ultrasensitive signal-response curves created by multisite phosphorylation. For the model simulation, we created a system of ordinary differential equations describing the phosphorylation of a set of kinase substrates carrying different multisite phosphorylation networks. The differently colored lines indicate simulated phosphorylation output responses at different thresholds of the increasing input signal of the kinase (the diagonal dotted line). The number of phosphorylation sites was varied in each substrate to gain temporal resolution of the response curves (1, 4, 6, 9, and 12 phosphorylation sites in substrates represented by curves from left to right, respectively). In addition, the phosphorylation rate constants of selected steps were varied in some substrates to imitate the effect of different patterning of the sites on the phosphorylation rates on individual steps. Weak phosphatase activity was included to counteract the kinase activity. The dotted horizontal lines represent the kinase thresholds at which half of the substrate has reached the fully phosphorylated state.

specific sites on the cyclin interact with docking motifs on the substrate (Holt *et al.*, 2009); and the small adaptor protein Cks1 interacts with specific phosphorylated threonines on the substrate (Tyanova *et al.*, 2013).

Analysis of the phosphorylation dynamics of Sic1 showed that the phospho-adaptor Cks1 is a key factor that controls the dynamics of multisite phosphorylation (Figure 1A). Cks1 binds to Cdk1 and also to phosphorylated sites, thereby enhancing the phosphorylation of neighboring sites positioned C-terminally at optimal distances from the Cks1-bound phosphorylated site. This type of

event occurs several times, leading to sequential phosphorylation of the entire network of sites and thereby leading to the phosphorylation of output degron sites at the C-terminal end of the network. Because clusters of sites are located in disordered segments of the proteins or in entirely disordered Cdk1 targets (Figure 1B), the cyclin-Cdk1-Cks1 complex acts as a catalytic scaffold whose net rate of catalysis of multisite phosphorylation through the network is determined by how well the fixed spatial orientation of three docking pockets on the scaffold fits with the linear pattern of phosphorylation sites and docking sites in the substrates.

Intriguingly, the sequential steps of phosphorylation in Cdk1 targets were found to propagate in an N-to-C terminal direction along the disordered polypeptide chain of the substrate. Furthermore, the productive interaction of a substrate with the cyclin-Cdk1-Cks1 scaffold was found to be defined by a set of crucial parameters that control the overall N-to-C terminal flux of multisite phosphorylation: 1) the choice of serine or threonine as the phospho-adaptor residues (only phosphothreonines serve as docking sites for Cks1); 2) the distance between sites along the disordered peptide chain; 3) the distance between the cyclin-specific docking sites and the phosphorylation sites; 4) the consensus binding motif of the phospho-adaptor subunit Cks1; 5) the overall number of phosphorylation sites; and 6) the processivity at each phosphorylation step. These findings show that the surrounding consensus specificity of the phosphorylation sites has only limited influence on the phosphorylation of each network. Instead, a simple set of rules, or a “multisite phosphorylation code,” defines the overall pattern and rates of phosphorylation in a multisite cluster, including its key output sites.

WHAT IS THE ADVANTAGE OF MULTISITE NETWORKS OVER A SINGLE-SITE PHOSPHORYLATION SWITCH?

It has been suggested that multisite phosphorylation of proteins could be the mechanism that processes a graded input signal of a protein kinase into an ultrasensitive, switch-like response (Ferrell, 1996; Harvey *et al.*, 2011; Trunnell *et al.*, 2011). In the simplest case, this mode of signal processing requires that the output signal of the switch corresponds to the fully phosphorylated state of the multisite target protein. In the presence of an opposing phosphatase activity, low levels of kinase input signal produce a relatively low fraction of target molecules in which all sites are phosphorylated. At higher input signals, fully phosphorylated molecules begin to accumulate, and the overall signal-response profile shows a more switch-like or digital shape compared with the hyperbolic shape expected from a single-site switch (Ferrell 1996; Harvey *et al.*, 2011; Trunnell *et al.*, 2011). This result can be obtained either with a randomly ordered series of phosphorylation sites or when sites are phosphorylated in a specific order, as described for Cdk1 targets. In the latter case, a low-level input signal would not produce a sufficient number of phosphorylated priming sites that are required for phospho-adaptor docking and efficient phosphorylation of the C-terminal output sites. At higher levels of the kinase input signal, phosphorylation of the output sites is amplified, and the overall shape of the response begins to resemble a digital step function.

DIFFERENT MULTISITE NETWORKS CAN PROVIDE DISCRETE RESPONSES TO DIFFERENT KINASE SIGNAL THRESHOLDS

Ultrasensitive response profiles may provide yet another important quality of the cyclin-Cdk1-Cks1 scaffold: they could help process different levels of increasing Cdk1 signal into discrete, temporally resolved events of the cell cycle. Different multisite phosphorylation networks have different Cdk1 signal processing capacities. The phosphorylation rates are controlled at each step by the network parameters described here earlier, and different substrates could support networks with different numbers of steps and a different set of rates. The output sites of some substrates are phosphorylated early in the cell cycle at lower Cdk1 thresholds (Figure 1C). At these low kinase levels, the Cdk1 signal is processed much less efficiently through the networks of later substrates, resulting in the output sites being weakly phosphorylated. Higher Cdk1 levels are required to push a critical amount of the signal through the network of later substrates for a sufficiently phosphorylated output (to use an elec-

tronics analogy, the networks of later substrates have more resistance). In this manner, different well-resolved Cdk1 thresholds in the cell cycle could be achieved. Thus the networks of phosphorylation sites in Cdk1 targets may act as “timing tags” by coding different patterns of sites.

DOCKING VIA PRIMED PHOSPHORYLATION SITES CAN CREATE SPECIFICITY FILTERS

Intriguingly, our studies of Cdk1-dependent multisite phosphorylation revealed that phospho-adaptor-mediated docking at primed phosphorylation sites enables Cdk1 to phosphorylate residues that are not CDK consensus sites (Koivomagi *et al.*, 2011a). In general, CDK target sites contain either optimal consensus phosphorylation motifs (S/T-P-x-K/R) or suboptimal consensus sites (S/T-P; Songyang *et al.*, 1994; Mok *et al.*, 2010). However, docking via optimally positioned and primed phosphorylation sites promotes the efficient phosphorylation of secondary sites that lack the required proline in position +1 (Figure 2A). This phenomenon could prove to be very important in Cdk1 signaling because it works as a filter to prevent other proline-directed kinases from erroneously triggering these Cdk1-dependent switches. In the case of both Sic1 and another Cdk1 target Far1 (unpublished results), the non-CDK site is part of the phosphodegron (output signal). Thus the multisite network allows the phosphorylation of the output degron by Cdk1 but not by other proline-directed kinases that do not have the cyclin-Cdk1-Cks1-like scaffold structure and thus cannot read the multisite phosphorylation code. Indeed, many kinases recognize the minimal consensus sequence S/T-P, which highlights the importance of this specificity filter. The output signals of Cdk1 targets must be kept very low to prevent both premature and partial signaling via Cdk1 switches in G1, as the leaking signals could induce genomic instability (Lengronne and Schwob, 2002).

SEQUENTIAL PHOSPHORYLATION OF SITES IN A NETWORK BY DIFFERENT KINASES CAN CREATE LOGIC GATES

Sequential processing of kinase signals via multisite networks is not limited to Cdk1. A recent study demonstrates that different kinase inputs can phosphorylate networks of sites in a specific order that is predetermined by the linear pattern of the sites in Eco1, an enzyme that performs the acetylation of cohesin in S phase (Lyons *et al.*, 2013). Degradation of Eco1 at the end of S phase depends on sequential phosphorylation of three sites by three kinase inputs (Cdk1, Cdc7-Dbf4, Gsk-3/Mck1; Figure 2B). The resulting phosphorylated configuration corresponds to a diphosphodegron recognized by the SCF-Cdc4 ubiquitin ligase. By using a sequential priming mechanism such as this, the multisite system acts as a three-branched AND gate that ensures that a sufficient level of each input kinase is present before the commitment of the switch. It is highly likely that this principle is widely used in cellular systems and that other logic functions are built by multiple kinase inputs.

One additional mechanism for generating complex logic functions could involve phosphorylation sites that negatively affect the phosphorylation of other sites in the same network. Indeed, we found evidence for this possibility in our studies of the Cdk1 targets and inhibitors Far1 and Sic1. Mitogen-activated protein (MAP) kinases that mediate external stimuli (osmotic stress, pheromones) influence Cdk1 multisite networks by a mechanism we call “diversion” (Valk and Loog, unpublished results). In this scheme, the binding of Cks1 to a MAP kinase-primed phospho-site diverts the Cdk1 complex to an alternative inhibitory network of sites, away

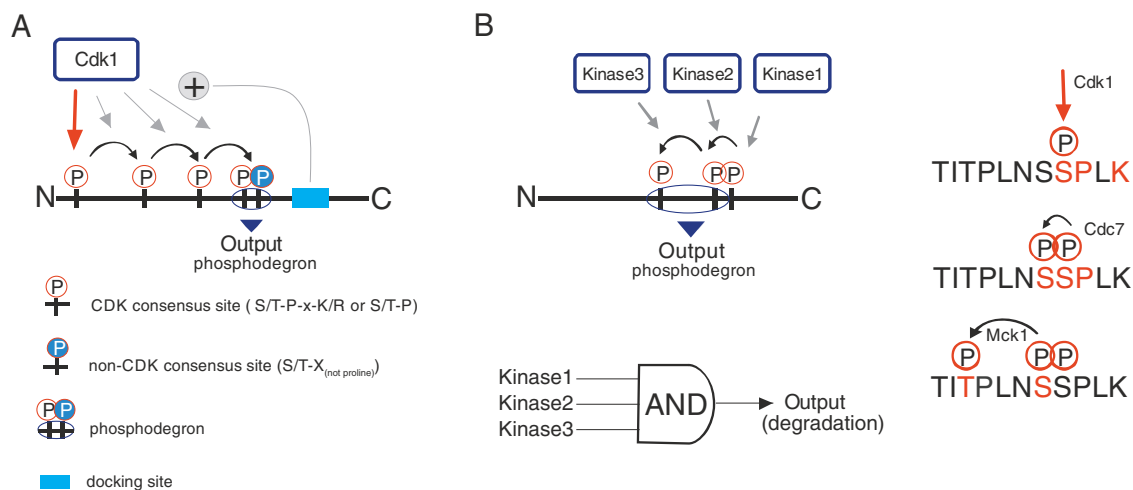


FIGURE 2: Sequential processing of kinase signals along linear multisite networks. (A) Schematic view of a specificity filter based on sequential phosphorylation of a substrate by cyclin-Cdk1-Cks1. The output signal is a diphosphodegron containing a non-CDK site. This system both prevents erroneous firing of the degradation switch by other proline-directed kinases and filters low-level Cdk1 signals to ensure that the commitment of the switch occurs only when adequate levels of Cdk1 activity are achieved. (B) A three-input AND gate created by sequential primed kinases that leads to the degradation of cohesin acetyltransferase Eco1 (Lyons *et al.*, 2013). The three kinases recognize motifs primed by previous phosphorylation events (motifs are indicated by red letters on the scheme at right). The resulting ordered sequence of three phosphorylation events leads to the formation of a phosphorylated diphosphodegron.

from the phosphorylation of sites that are responsible for the cell-cycle-dependent degradation of Far1 or Sic1. Thus the strength of the MAP kinase input determines the fraction of free Cdk1 complex available for generating a signal that triggers entry into the cell cycle and the initiation of S phase. These and other potentially undiscovered logic schemes in multisite phosphorylation networks may provide the cell with the capacity to execute complex computations of responses to external and internal signals. The number of phosphorylation steps in a switch and the complexity of multisite networks can also be increased by combining the phosphorylation of several targets with the assembly of protein complexes. The assembly steps of the complexes could trigger sequences of new phosphorylation events because different subunits in the complex could provide docking interactions for the phosphorylation of other subunits.

PERSPECTIVE: MULTISITE PHOSPHORYLATION NETWORKS CAN BE USED AS TUNABLE COMPONENTS OF SIGNALING CIRCUITS IN SYNTHETIC BIOLOGY

Nature's ubiquitous use of multisite phosphorylation systems could inspire us to apply this logic to the design of signaling circuits for a diverse range of synthetic biology applications. In the era of affordable DNA synthesis and genome-editing tools, creating a toolbox of easily tunable multisite phosphorylation tags may become useful in synthetic systems. The flexibility of multisite networks will enable us to design synthetic protein segments that encode different kinase specificities and output phosphorylation patterns. It is highly likely that future studies will reveal more multisite phosphorylation rules for different kinases and that this knowledge will help us to design additional signal-response modules, including logic gates, oscillators, memory circuits, single-input modules with multiple kinase thresholds, and so on.

To facilitate the flexible design of circuits from base components, it is possible to introduce engineered kinase inputs by linking kinase domains (with basophilic, acidophilic, and proline-directed substrate specificity) to various phospho-adaptor modules (Cks1, 14-3-3, Polo

box, etc.). The resulting chimeric kinases would enable the sequential processing of kinase signals via networks that contain phosphorylation sites with different consensus motifs.

Using interdependent phosphorylation events within networks of phosphorylation sites in proteins, cellular systems have acquired a vast computational capacity whose diverse possibilities are not well understood. Further studies will likely reveal more schemes for encoding logic using multisite networks, and it is also likely that the knowledge gained will offer insights into how this technique could be applied in the design of signaling circuits within synthetic cells.

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