

Multisite Phosphorylation and the Countdown to S Phase

Minireview

Raymond J. Deshaies^{1,3} and James E. Ferrell, Jr.²

¹Division of Biology and Howard Hughes

Medical Institute

California Institute of Technology

Pasadena, California 91125

²Departments of Molecular Pharmacology and Biochemistry

Stanford University School of Medicine

Stanford, California 94305-5174

Remarkably, SCF^{Cdc4} ubiquitin ligase binds and ubiquitinates Sic1 decorated with six, but not five, phosphates (Nash et al., 2001). This numerical wizardry suggests how analog inputs can be rectified to digital outputs. Unraveling the counting mechanism promises to generate new insights into the architecture of protein nanoprocessors.

Sic1 and the G1/S Transition

Sic1 and the B-type cyclin (Clb)-cyclin-dependent kinase (CDK) complexes that drive S phase and M phase in budding yeast are locked in mortal combat over control of the cell cycle (Deshaies, 1997). Sic1 inactivates Clb-CDK complexes and promotes Clb degradation, whereas Clb-CDKs antagonize Sic1 transcription and promote Sic1 degradation. As cells exit mitosis, Clb-CDK activity declines and a bolus of Sic1 is produced. Sic1 persists throughout G1 phase, and prevents the precocious activation of DNA synthesis by Clb-CDK complexes that assemble in G1 cells (Schwob et al., 1994; Schneider et al., 1996). Although Sic1 is rock-stable during extended periods of G1 arrest, its degradation commences in earnest once cells commit to cell cycle entry at START and negotiate the G1/S transition (Figure 1). The control step in Sic1 degradation is its phosphorylation by Cln-CDK, which is necessary and sufficient to trigger binding of Sic1 to constitutively active SCF^{Cdc4} ubiquitin ligase, which then ubiquitinates Sic1, thereby targeting it for degradation by the 26S proteasome (Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997). This leads to emancipation of active Clb-CDK (Verma et al., 2001), which goes on to stimulate DNA synthesis (Schwob et al., 1994). Phosphorylation, ubiquitination, and degradation of Sic1 can be completely reconstituted with purified components (Verma et al., 2001), and thus a key aspect of the G1/S transition—abrupt activation of Clb-CDK by Cln-CDK—can be dissected with a sophisticated array of biochemical and genetic tools. This prior work sets the stage for a key question: is the G1/S transition a sharp, all-or-none demarcation between biochemically distinct G1 and S phases, and if so, how is a switch-like transition crafted from the reactions described above?

Sic1 Must Be Phosphorylated on Multiple CDK Sites to Be Targeted to SCF^{Cdc4}

Prior studies on Sic1 phosphorylation revealed that successive elimination of CDK sites progressively dimin-

ishes the ability of Cln-CDK-treated Sic1 to serve as a substrate for SCF^{Cdc4}, and mutants lacking four CDK sites (Sic1- Δ 4P) are no longer ubiquitinated by SCF^{Cdc4} or degraded (Verma et al., 1997). Nash et al. (2001) initiated their intriguing study by performing the flip-side experiment: all nine consensus CDK phosphorylation sites in Sic1 were converted to alanine to yield Sic1+0P, and then single sites were restored one by one. Mutants were assayed for their ability either to bind Skp1-Cdc4 in vitro after phosphorylation by Cln-CDK, or to arrest cell proliferation when overexpressed from the GAL promoter. The latter assay is based on the observation that overexpression of nondegradable Sic1- Δ 4P arrests cells at the G1/S boundary with negligible Clb-CDK activity (Verma et al., 1997). Nash et al. restored phosphorylation sites back to Sic1+0P in order of their importance, with the site whose elimination evokes the greatest inhibition of Sic1 turnover added back first and so on. Remarkably, readdition of the five (out of nine total) seemingly most important CDK sites does not restore binding to Cdc4 or curb the toxicity of overproduced Sic1. This dovetails nicely with the fact that three different Sic1- Δ 4P mutants (that each retain five CDK sites) are poor substrates for SCF^{Cdc4} in vitro (Verma et al., 1997). The astonishing result that propels Nash et al. into the limelight is that readdition of a sixth CDK site—a seemingly innocuous serine at either amino acid 69 or 80—abruptly restores the ability of Sic1 to bind Cdc4 and abrogates the lethal effect of Sic1 overexpression. Because (1) restoration of either of two CDK sites has the same “light switch” effect in both assays, and (2) the S69 and S80 sites are not particularly significant to Sic1 turnover as judged from analysis of single mutants, Nash et al. conclude that it is the presence of six phosphorylations (and not the identity of the sixth phosphorylation site) that governs Sic1’s ability to serve as a substrate for SCF^{Cdc4}.

In the first of a long trail of steps they took to explore this surprising result, Nash et al. characterized the nature of the Sic1 epitope that Cdc4 binds. Although phosphopeptides that span single CDK phosphorylation sites within Sic1 bind very poorly to Cdc4, a phosphopeptide that contains the conserved threonine 380 phosphorylation site of cyclin E binds with a respectable K_d of 1 μ M. T380 helps specify ubiquitination of cyclin E by the recently discovered SCF^{H^{Cdc4}/Fbw7} complex (Koepp et al.,

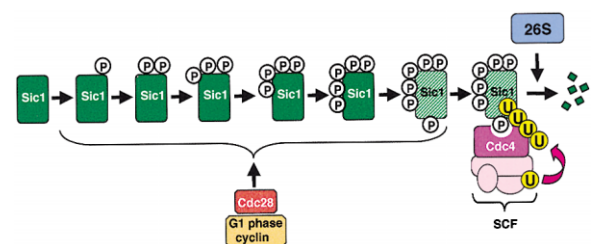


Figure 1. Multisite Phosphorylation Enables Sic1 Degradation at the G1/S Transition in Budding Yeast

See text for details.

³Correspondence: deshaies@its.caltech.edu

2001; Moberg et al., 2001; Strohmaier et al., 2001). A far-Western analysis that employed a macroarray of cyclin E peptide variants fixed to a filter revealed an optimal phosphopeptide ligand for Cdc4: L/I-L/I/P-pT-P-<RK>₄ (R and K disfavored in the +1 through +4 positions). Intriguingly, whereas the cyclin E T380 phosphopeptide is an excellent ligand for Cdc4, CDK sites in general—and those of Sic1 in particular—are embedded in sequences that render them poor ligands. This counterintuitive observation spawned the idea that six phosphorylations on Sic1 may act like six tiny strips of Velcro that individually form weak bonds, but together enable a stable link between Sic1 and Cdc4. By contrast, the high affinity phosphopeptide within cyclin E is like a dab of super-glue that by itself can affix substrate to Cdc4.

To test the “Velcro versus glue” idea, Nash et al. did two distinct experiments. First, they constructed artificial fusion proteins that consist of concatamerized phosphopeptide sequences from Sic1 fused to glutathione-S-transferase (GST). Purified chimeras were treated with Cln-CDK and then tested for binding to Cdc4. GST fused to 3 or 6 consecutive copies of Sic1-derived peptides does not bind Cdc4, whereas chimeras that contain 9 or 12 peptide repeats bind tightly. In the second experiment, the S76 phosphorylation site and surrounding sequences were mutated to match the sequence of the optimal Cdc4-phospho degnon (CPD), gleaned from the peptide macroarray studies. The resulting mutant, Sic1^{CPD}, was engineered so that the CPD is the sole CDK target. Remarkably, CDK-treated Sic1^{CPD} binds SCF^{Cdc4} and is ubiquitinated *in vitro*, and is not toxic to yeast cells upon overexpression *in vivo*. Taken together, these experiments support the hypothesis that either one drop of super-glue or six tiny strips of Velcro can suffice to direct Sic1 degradation via SCF^{Cdc4}.

Why use six strips of Velcro when one drop of super-glue will do? To address this important question, Nash et al. expressed Sic1^{CPD} in place of normal Sic1 in budding yeast cells. Sic1^{CPD} is present at lower levels than natural Sic1 in pre-START cells, possibly due to precocious degradation. Moreover, during progression from G1 into S phase, Sic1^{CPD} is destroyed earlier than Sic1, and consequently *SIC1^{CPD}* cells enter S phase prematurely. As is observed for *sic1Δ*, premature entry into S phase in *SIC1^{CPD}* cells wreaks havoc on the genome, resulting in a 100-fold increase in chromosome loss.

Nash et al. conclude their paper by gathering together their diverse collection of observations into a simple and elegant hypothesis. They argue that a requirement for six phosphorylations sets a threshold for Cln-CDK activity, such that at low levels of Cln-CDK, Sic1 is stable, but as Cln-CDK levels escalate, Sic1 degradation is activated in a switch-like fashion. This intriguing idea provides a penetrating glimpse into how regulatory proteins can be wired together to generate a decisive cell cycle transition.

As is often the case for work that stretches the existing paradigms, this paper contains some unresolved issues and leaves numerous interesting questions to tackle in the future. We'll quickly dispense with some caveats, and devote the remainder of this review to some interesting ramifications that might be profitable subjects for future investigations. First, although Sic1 degradation is clearly switched on by addition of a sixth consensus

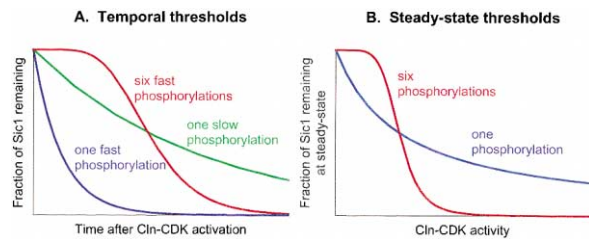


Figure 2. Multisite Phosphorylation and Switch-like Responses

(A) Temporal thresholds. The red curve represents a calculated time course for Sic1 destruction, assuming that Sic1 destruction is triggered by six equally rate determining phosphorylations. The other curves show Sic1 destruction if it is triggered by one fast (blue) or slow (green) phosphorylation.

(B) Steady-state thresholds. Sic1 concentration as a function of Cln-CDK activity if degradation is triggered by one (blue curve) or six (green curve) phosphorylations. Modeling was carried out with Mathematica 2.2.2.

CDK site, it is not necessarily true that six phosphorylations are required (see below, When Does 6 = 6?). Second, it is not established that the Sic1+5P and Sic1+6P mutants are actually degraded at significantly different rates *in vivo*; half-life measurements would be instructive. Third, the reduced level of Sic1^{CPD} in G1 cells (e.g., *cdc28-13* mutants) is a matter of concern, because it suggests that a fraction of Sic1^{CPD} molecules may be inappropriately rerouted for degradation by a protein kinase that does not normally participate in Sic1 turnover. Because growth is restored to diploids bearing homozygous deletions for all three *CLN* genes by deleting a single copy of *SIC1* (Schneider et al., 1996), it stands to reason that the diminished level of Sic1^{CPD} in G1 cells may fall below a critical threshold that establishes the normal requirement for Sic1 degradation. If Sic1 levels indeed decline in early G1 phase by a Cdc28-independent pathway, it would beg the question of whether multisite phosphorylation of Sic1 by Cln-CDK is really required for high fidelity chromosome transmission. It is worth noting that overexpression of G1 cyclin, which advances the timing of Sic1 turnover, can have minimal impact on the fidelity of chromosome transmission (Vallen and Cross, 1995).

Does Multisite Phosphorylation Enable a Switch-like Destruction of Sic1 at G1/S?

These caveats notwithstanding, why did evolution sculpt the G1/S transition such that multisite phosphorylation is required to eliminate Sic1? Wouldn't an optimally tuned single phosphorylation site (e.g., one that is phosphorylated more slowly than the CPD site) work just as well? Nash et al. suggest a potential advantage of multisite phosphorylation based on simple kinetic considerations. Suppose that six equivalent phosphorylations are needed to trigger Sic1 destruction. Then Sic1 destruction will initially be very slow, while the first five sites are being phosphorylated, and after a lag period will increase more rapidly (Figure 2A, red curve). The result is that a temporal threshold is built in to the destruction of Sic1. Now suppose that Sic1 destruction is driven by a single phosphorylation. There will no longer be a lag period between Cln-CDK activation and Sic1 destruction (Figure 2A, blue curve), even if the phosphor-

ylation of Sic1 is slowed down (Figure 2A, green curve). Thus, multistep phosphorylation can build a time delay into the degradation of Sic1.

In addition, a requirement for multisite phosphorylation could allow Sic1 to ignore low levels of Cln-CDK activity in early G1 phase, and then respond decisively once Cln-CDK activity has exceeded a threshold level. Because formation of hexa-phosphorylated Sic1 could potentially be proportional to $[\text{Cln-CDK}]^6$, it is easy to envision how an ultrasensitive switch-like response might ensue (Figure 2B). A similar argument has been made for the switch-like activation of p42 MAP kinase by MEK in *Xenopus* oocytes. In that case, the distributive phosphorylation (i.e., each phosphorylation involves an independent collision between enzyme and substrate) of two sites on MAP kinase gives rise to a slightly ultrasensitive response, which is then amplified into a highly ultrasensitive response by the architecture of the MAP kinase cascade (Huang and Ferrell, 1996; Ferrell, 1997). If six distributive phosphorylations indeed target Sic1 for degradation, this could generate a highly ultrasensitive response without the need of a cascade.

The ultrasensitivity generated by multistep phosphorylation of Sic1 might be amplified into an even more switch-like response—a bistable response—by positive feedback. Indeed, Clb-CDK can phosphorylate Sic1 and thus liberate more Clb-CDK complexes, providing a positive feedback loop from which bistability could arise. This is exactly what happens in the frog oocyte MAPK cascade, where MAPK activates translation of the first kinase in the cascade, thereby converting a “button switch” (which requires continuous application of signal to remain on) into an irreversible toggle switch (Ferrell and Machleder, 1998). However, experimental evidence suggests that activation of Clb-CDK is not required to sustain normal Sic1 turnover at the G1/S transition (Verma et al. 1997).

How Does Cdc4 Count to Six?

In proposing that multisite phosphorylation of Sic1 sharpens the G1/S transition into a switch, Nash et al. predict that the ubiquitination of Sic1 by SCF^{Cdc4} (and subsequent degradation) should abruptly turn on as Cln-CDK activity is progressively increased. This is a key prediction that should be readily testable given the available technology (Verma et al., 2001). If their prediction holds true, it raises a fundamental question about protein design: how might evolution sculpt a receptor that monitors hexaphosphorylation of its ligand?

One simple counting mechanism would be for Cdc4 to have six phosphopeptide binding sites. However, three lines of evidence presented by Nash et al. argue for a single phospho-Sic1 binding site per molecule of Cdc4. First, Scatchard analysis and Hill plots of binding data reveal only one class of phosphopeptide binding site on Cdc4 and no evidence of enthalpic cooperativity. Second, a single phosphopeptide derived from cyclin E competes with multiply-phosphorylated Sic1 for binding to Cdc4. Third, three conserved arginine residues in the WD-40 domain that are required for the phosphopeptide binding activity of Cdc4 are predicted by structural modeling to form a single pocket. The postulated existence of a single phosphopeptide binding site on Cdc4 begs the question of why adding a sixth phosphorylation site

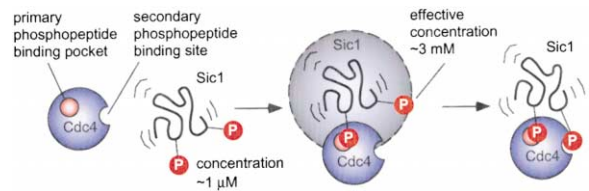


Figure 3. Induced Proximity and the Binding of Sic1 Phosphopeptides to Secondary Sites on Cdc4
See text for details.

to Sic1 should augment its binding to Cdc4 by far more than 20%.

But maybe an SCF^{Cdc4} complex can recognize more than one phosphate at a time after all. There are at least three possible ways to envision this. First, the phosphopeptide binding pocket may accommodate more than one phosphopeptide at a time. Note that the Scatchard analysis does not exclude the existence of multiple comparably strong sites that bind phosphopeptides noncooperatively, or weak secondary binding sites. Second (as suggested by the authors), Cdc4 may function as an oligomer (Kominami et al., 1998; Wolf et al., 1999), with each Cdc4 monomer contributing a binding site (or sites). Third, Cdc4 may possess one or more secondary phosphopeptide binding sites in addition to the arginine-lined pocket. The basic idea is illustrated in Figure 3, where we show how bisphosphorylated Sic1 might interact with two binding sites on a monomeric Cdc4. The first phosphate binds the arginine pocket on Cdc4. Given that Sic1 is highly flexible (Nash et al. 2001), one can imagine the second phosphorylated residue flopping around within a sphere whose radius is ~ 5 nm. One phosphopeptide per 5 nm sphere translates to an effective concentration of about 3 mM—a 3000-fold increase over the normal concentration of Sic1 in budding yeast cells ($\sim 1 \mu\text{M}$). Thus, the binding of the first Sic1 phosphopeptide to the arginine pocket tethers the second phosphopeptide in such close proximity to Cdc4 that even a very weak binding interaction becomes favorable (i.e., entropic cooperativity). In considering potential secondary binding sites, it is worth noting that other amino acids besides arginine can make energetically favorable contacts with phosphate (Lu et al., 1999).

Any of these mechanisms—multiple phosphates occupying the arginine pocket, Cdc4 oligomerization, or secondary binding sites—acting separately or in combination, could allow an SCF^{Cdc4} complex to bind simultaneously to multiple phosphopeptides on Sic1. But still, we are left with the question of why a sixth phosphorylation event appears to be so crucial. Here, the solution could lie in the relationship between binding energy and dissociation constants. The dissociation constant is proportional to the logarithm of the binding energy, not to the binding energy itself. Thus, each phosphate could decrease the K_d by a multiplicative factor of 2 or 10 or 100, and the difference in how much complex can be formed by Sic1+5P versus Sic1+6P at physiological Cdc4 concentrations could be considerable.

When Does 6 = 6?

The apparent requirement for six phosphorylation sites does not necessarily mean that Sic1 must be hexa-

phosphorylated to bind Cdc4. Fewer than six phosphate groups per molecule of Sic1 may suffice to constitute a targeting signal, but some of the reintroduced sites may be inefficiently phosphorylated for a variety of reasons. Thus, it may take six sites simply to ensure incorporation of the requisite number of phosphates. If phosphorylation of Sic1 is likened to throwing darts, the readdition of six sites simply makes for a bigger bull's-eye for Cln-CDK to aim at. For sake of argument, we hypothesize that the true degron comprises four phosphorylations. If so, there would be only one way to form a stable complex between Cdc4 and a Sic1 molecule that contains four phosphorylation sites. If Sic1 contains five sites, there are 5 different ways to form quadruply phosphorylated Sic1 (and one way to form Sic1+5P), resulting in a potential increase in binding affinity of 6-fold. If Sic1 contains six sites, there are $6!/4!2! = 15$ different possible configurations of Sic1+4P, 6 configurations of Sic1+5P, and Sic1+6P (22 total). Thus, increasing the number of phosphorylation sites from 4 to 6 can enhance the statistical likelihood of generating a complex between quadruply phosphorylated Sic1 and Cdc4 by 22-fold! This idea—a sort of combinatorial cooperativity—should be readily testable by applying a collection of heterogeneously phosphorylated Sic1 molecules to a Cdc4 affinity resin, and using mass spectrometry to deduce the mass of the phospho-Sic1 species that are retained on the matrix (Annan et al., 2001).

Nanobioprocessor Technology: There's Plenty of Room at the Bottom

Now that we know that Cdc4 can count, it will be fascinating to learn how high it can count, and how it goes about counting. Although the design of Cdc4's abacus currently remains beyond our grasp, you can surely count on one thing: Cdc4 isn't the cell's only accountant with such a subtle talent for enumeration. It is well-known that CDK substrates commonly are phosphorylated on multiple sites, and a recent study revealed that multiple CDK sites on subunits of three distinct protein complexes govern the block to rereplication of a cell's chromosomes during a single round of division (Nguyen et al., 2001). Although the presence of multiple CDK sites in substrates has often been interpreted as evidence for redundant control that renders regulation more robust, an alternative hypothesis is that the presence of multiple CDK sites in substrates serves to tune downstream responses to spatial or temporal gradients of CDK activity. A major challenge for the future will be to see how many "nanobioprocessors" akin to Cdc4 are embedded in the cell's circuitry, and how they are wired together to calculate a cell's biology.

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