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Review

Regulatory modules: Coupling protein stability to phopshoregulation during cell division

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ABSTRACT

Multiple post-translational regulation systems regulate cell biology. Two key mechanisms that coordinate the myriad processes of cell replication are phosphorylation and ubiquitin-mediated degradation of proteins. Regulatory modules have evolved to integrate these two control systems at key decision points in the cell division cycle. These modules enable information to be processed with high fidelity by filtering noise, improving specificity, generating feedback loops, and optimizing spatiotemporal coordination of cellular processes. This review provides examples of these modules and considers the advantages of this signaling nexus.

Regulated degradation and phosphorylation are complimentary mechanisms that modulate the activity of proteins; integration of these mechanisms provides robust control. This review will consider two modules that link phosphorylation to protein stability. The first is the phosphodegron, a short linear motif that is activated by the addition of one or more phosphate groups. Here, phosphorylation drives instability (Fig. 1A). The second is the combination of a constitutively active destruction motif with phosphorylation motifs such that phosphorylation masks the destruction signal. This module has the opposite logic: phosphorylation stabilizes an otherwise unstable molecule (Fig. 1B). Herein, I will refer to this combination of motifs as the phospho-inhibited degron. I will consider a handful of examples of each of these modules and explore how their employment enables faithful control of cell division.

1. The rise and fall of Cyclins

In the early 1980s, researchers at the Woods Hole Marine Biology labs observed the periodic destruction of proteins at each cell division [1]. We now know that regulated proteolysis is a key mechanism that coordinates cell division in all Eukaryotes.

The periodically degraded proteins were the Cyclins, critical co-activators of the Cyclin-Dependent Kinases (CDKs). CDKs are the major coordinators of the various cell biological events that constitute cell duplication and division. The rise and fall of CDK activity leads to spatial and temporal fluctuations in the phosphorylation state of hundreds of proteins, thereby ordering the events of the cell division cycle (for a comprehensive overview see [2]).

In the first phase of the cell cycle, G1, CDK levels are low and cells accumulate biomass. When sufficient growth has occurred and the correct signals are present, CDK levels rise to an intermediate level. This transition from low to intermediate CDK activity is dependent on the destruction of a stoichiometric inhibitor of CDK and will be discussed in detail below. Upon reaching intermediate activity, CDK phosphorylates a cohort of proteins, activating many processes (DNA and organelle replication) and inactivating others (licensing of DNA replication). These processes are referred to as S (synthesis) phase.

When DNA replication is completed, CDK activity rises further, and a larger group of substrates become phosphorylated leading to spindle assembly, chromosome condensation, and the preparation of the cell for division at metaphase.

Progression from metaphase to anaphase and subsequent cytokinesis requires the inactivation of Cdk1 and resultant dephosphorylation of proteins. This inactivation is driven by two major mechanisms: the activation of the Anaphase Promoting Complex/Cyclosome, which targets Cyclins for destruction; and the

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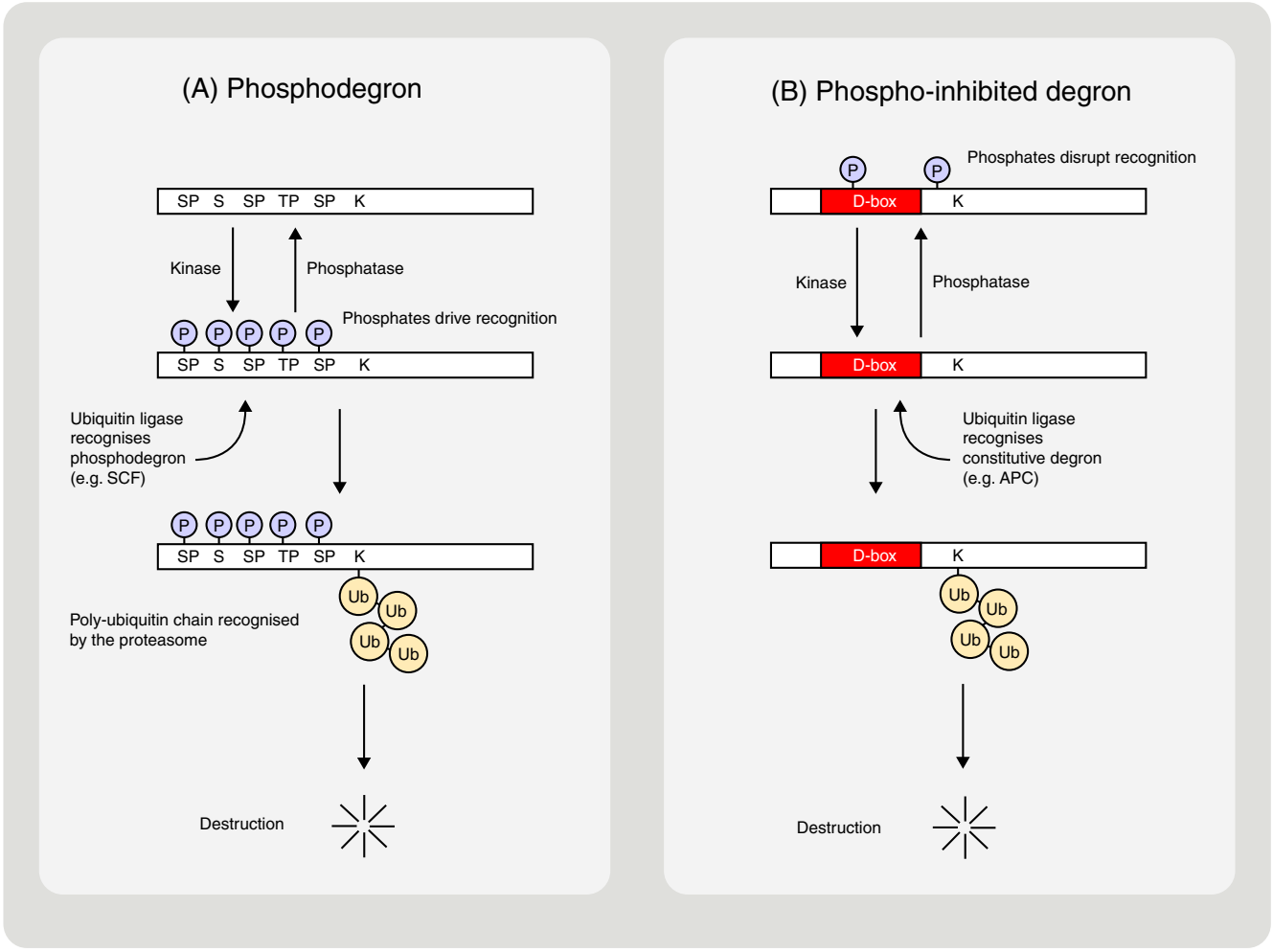

Fig. 1. Regulatory modules that couple protein stability to phophorylation. (A) The phosphodegron: A kinase phosphorylates residues within a specific sequence context to generate a phosphopeptide that binds to a ubiquitin ligase. In the absence of phosphorylation, the protein is stable. Upon phosphorylation, the protein is degraded. (B) The phospho-inhibited degron: A degradation signal (degron) is recognized by a ubiquitin ligase. Phosphorylation of residues within and around the degron disrupts the interaction between degron and ubiquitin ligase. Upon phosphorylation, the protein is stabilized.

accumulation of stoichiometric inhibitors of CDK, a process modulated by the Skp1-Cullin-F-box ligase.

2. Arbiters of destruction: the Skp1-Cullin-F-box ligase and the Anaphase Promoting Complex

Protein degradation is an active and highly regulated process. The molecule at the core of this regulation is ubiquitin, a small, highly conserved protein. Ubiquitin is conjugated to the primary amines of lysine residues, and at the N-terminus of proteins. Ubiquitin contains multiple lysines, and therefore can be conjugated onto itself forming poly-ubiquitin chains of various structures. Poly-ubiquitin chains are recognized by the proteasome, leading to the destruction of proteins carrying this signal (reviewed in [3]).

Ubiquitination is catalyzed by ubiquitin ligases. Two ubiquitin ligases are central to the control of protein degradation during cell division: the Skp1-Cullin-F-box ligase (SCF) and the Anaphase Promoting Complex/Cyclosome (APC/C). For a detailed description of the structure and regulation of these ligases see [4–6]. This review will focus on the short linear motifs that mark proteins for destruction and how these motifs are generated or modulated by phosphorylation.

3. Part 1. The phosphodegron module

The phosphodegron is a short linear motif that is inert until phosphorylated, but upon phosphorylation generates a binding surface that interacts with a ubiquitin ligase. The main ubiquitin ligase that recogises phosphodegrons in the cell cycle is the SCF [7,8]. The SCF is a multisubunit complex that consists of a core (Skp1, Cul1 and Rbx1), together with an F-box protein that binds to substrate molecules. The Cdc4 protein of Saccharomyces cerevisiae was the first F-box protein to be described [9]. This protein forms a WD40 fold that recognizes phosphorylated peptides [10]. Many other F-box proteins exist, and the majority of these recognize phosphorylated motifs [11].

4. Destruction of Sic1 is required for the G1–S phase transition

The most well studied phosphodegron is that of Sic1, an inhibitor of the Cyclin-Dependent Kinase Cdk1 in the model organism *Saccharomyces cerevisiae* [12]. Sic1 binds to the Cyclin-B/Cdk1 complex stoichiometrically and keeps it tightly inhibited, thereby preventing progression from G1 to S phase of the cell cycle. However,

there are several flavors of Cdk1; the G1-cyclin/Cdk1 complex does not bind to Sic1 and therefore is not inhibited. G1-cyclin/Cdk1 accumulates as the cell grows and begins to phosphorylate the Cdk1 consensus motifs (S/T*–P; where S/T* indicated the phosphoacceptor serine or threonine, and P represents proline) present at the N-terminus of Sic1. The phosphorylated Cdk1 consensus motifs on Sic1 bind to Cdc4, an F-box subunit of the SCF complex. The SCF then conjugates ubiquitin chains onto Sic1, thus targeting Sic1 for destruction at the proteasome [7,8]. As each molecule of Sic1 is destroyed, an active Cyclin-B/Cdk1 complex is released, that is capable of further phosphorylation of Sic1 molecules. Phosphorylation of Sic1 by Cyclin-B/Cdk1 leads to further Sic1 destruction and hence release of more Cyclin-B/Cdk1 in a positive feedback loop that ensures commitment to S phase.

5. Allovalency is a possible mechanism to generate switch-like protein degradation

The mechanism by which the Sic1 phosphodegron is generated and recognized have been elucidated over a series of studies that elucidate some important principles related to multi-site phosphorylation. The N-terminus of Sic1 contains not just one or two, but seven Cdk1 consensus sites. A 2001 study by Nash et al. [13] provided the first detailed exploration of the function of these clusters of phosphorylation sites. This study began with the determination of the preferred recognition sequence for Cdc4 as L/I–L/I/P–pT–P–{R/K}4, where L/I indicates preference for Leucine or Isoleucine, pT indicates a phosphothreonine and {R/K}4 refers to counter-preference for the basic amino acids arginine and lysine in the +2 to +5 positions. The phosphorylated Cdk1 sites in Sic1 do not conform to this ideal binding motif for Cdc4. The authors therefore hypothesized that synergy between multiple weak binding sites conferred by multiple phosphorylation sites ultimately allows binding of the phosphodegron to Cdc4 with sufficient affinity to allow poly-ubiquitination of Sic1 prior to escape.

If the kinase that generates this kind of degron acts in a distributive manner (i.e. each of the phosphates is added sequentially over the course of multiple binding and catalysis steps), there is a prediction of non-linearity in the system: the production of functional phopshodegrons will increase suddenly at a threshold of kinase activity. This cooperative binding of many weak sites was termed allovalency. Mathematical modeling demonstrates that it is possible to generate switch-like behavior using allovalent phosphodegrons [14]. The concept of allovalency is very interesting, especially in cell division where Cdk1 tends to phosphorylate substrates at clusters of multiple phosphorylation sites [15].

6. Processive phosphorylation cascades allow signaling specificity

Recent results from Koivomagi et al. [16] suggest that allovalency may not be at play in the case of Sic1 destruction. Exhaustive kinetic studies provide an alternate explanation for multi-site phosphorylation in the generation of the Sic1 phosphodegron. The authors found that the phosphorylation of Sic1 is not distributive but rather is highly processive (e.g. each time the kinase binds, multiple phosphates are transferred to Sic1 in multiple catalytic cycles).

The processivity of phosphorylation is due to the presence of a phosphate-binding module, Cks1, that is part of the Cdk1 complex. First Cdk1 transfers a phosphate, then the Cks1 subunit binds to that phosphate and directs the active site of Cdk1 to phosphorylate a nearby consensus site. Cks1 and Cdk1 are well-ordered, globular proteins [17] and therefore, one would expect there to be an optimal distance between the phoshopeptide bound by Cks1 and the subsequent phosphoacceptor site.

Cdk1 initially phosphorylates Sic1 at sites that do not form Cdc4-binding phosphopetides, but the Cks1 subunit of the Cdk1 complex can bind to these initial sites to enable a processive cascade of phosphorylation reactions that ultimately generates a phosphodegron. Sic1 appears to contain at least two phosphodegron modules consisting of two phosphorylated residues spaced four amino acids apart. This is consistent with the earlier idea that multiple sub-optimal phosphopeptides bind cooperatively to Cdc4. Indeed, structural studies have confirmed that Cdc4 contains two phosphate-binding surfaces [18].

Finally, this phosphodegron includes phosphopeptides that do not conform to a Cdk1 consensus site. However, Cdk1 is able to phosphorylate these non-consensus sites because Cks1 binds to priming sites and thereby positions the kinase active site at a very high local concentration next to the non-consensus site [16].

This study provides an interesting new way to think about multi-site phosphorylation and the generation of phosphodegron motifs. The basic consensus sequence for Cdk1 is very simple (S/T*–P) and overlaps with many other proline-directed kinases. In particular, the MAP kinases recognize the same consensus motif. Cross-talk between the MAP kinases and Cdk1 is undesirable since these signaling pathways lead to significantly different outcomes: Cdk1 drives S-phase, whereas the osmotic stress and mating pathway MAP kinases arrest division in G1. MAP kinases can phosphorylate S/T*–P motifs but MAP kinases are not processive and therefore are highly unlikely to generate the second component of the phosphodegron that does not conform to the S/T*–P consensus. The processive phosphorylation of multiple sites by Cdk1 can therefore be thought of as an information-processing event providing a mechanism to distinguish Cdk1 signals from those of the MAPK pathways.

7. Multi-kinase phosphodegrons integrate information

The formation of phoshodegrons by multiple phosphorylation sites creates opportunities for signal integration. More than one kinase can participate in the generation of such a phosphodegron thus generating logic gates that integrate information from multiple signaling pathways. For example, in vertebrates, Emi1 (Early Mitotic Inhibitor 1) degradation is due to the generation of a phosphodegron by the concerted action of Cdk1 and Polo kinase. Cdk1 first phosphorylates Emi1 creating a docking site for the phopshopeptide-binding domain of Polo. Polo binds to this priming site and then generates the phosphodegron of Emi1 [19,20]. Emi1 controls cell cycle progression by inhibiting the Anaphase Promoting Complex, therefore preventing progression to anaphase [21]. The Emi1 phosphodegron thus acts as an AND gate: both Cdk1 and Polo must be active to allow progression to anaphase.

8. Part 2. The phospho-inhibited degron module

The second major cell cycle ubiquitin ligase is the Anaphase Promoting Complex (APC). The core subunits of the APC are related to the SCF but the APC is a much larger complex consisting of 15 or more subunits, many of which are present in multiple copies, together weighing in at over a megadalton. The activity of the APC is tightly regulated during the cell cycle and oscillates more or less out of phase with Cdk1 activity. This regulation is complex and integrates a great deal of information about the state of the cell (reviewed in [4,5,22,23]). Again, I will not focus on APC regulation here, but rather focus on the degradation motifs that this enzyme recognizes.

The APC recognizes a diverse set of short linear interaction motifs. The best described is the Destruction box motif (D-box), minimally (R–x–x–L), the more complete consensus sequence being (R–x–x–L–x–x–x–x–N/D/E) [24]. The second best understood motif recognized by the APC is the eponymous KEN box (K-E-N) [25]. Several other unrelated sequences that the APC recognizes have also been described (e.g. [26,27]). These peptides do not require post-translational modification to bind to the APC, but are instead constitutive interaction modules. The APC is subject to a huge amount of regulation and so the stability of APC substrates is in large part a reflection of the activity state of this ubiquitin ligase. In addition, there are now several examples where APC interaction motifs are modulated by phosphorylation. Typically, phosphorylation disrupts the interaction between the APC and a D-box motif, thus masking the degradation motif. In the second part of this review I will present some examples of the phospho-inhibited degron module and its employment in the cell division cycle.

9. DNA damage leads to stabilization of securin, thus delaying anaphase

The onset of anaphase – the segregation of sister-chromatids in mitosis – is one of the most important and tightly regulated events of cell division. One key regulator of this process is securin, an inhibitory chaperone that binds to and inactivates the protease, separase. When conditions are appropriate, the APC recognizes a D-box motif in securin, leading to ubiquitination and destruction of the securin molecule. The destruction of securin releases separase, which then cleaves a member of the cohesin complex thereby dissolving the cohesion between chromatids, allowing them to be segregated by the spindle.

It would be catastrophic to attempt to segregate chromatids if they were not attached to the spindle, completely replicated and free of damage. The presence of DNA damage triggers a signaling pathway that culminates in the activation of the Chk1 kinase. Chk1 kinase phosphorylates consensus motifs near to the D-box of securin [28], thereby preventing recognition of this destruction motif by the APC [29]. Thus, in the presence of DNA damage, the destruction motif in securin is masked. The resultant delay in securin degradation provides an opportunity to repair DNA damage prior to anaphase.

10. Cdc6 is unstable until phosphorylation of its D-box, limiting the window of opportunity for licensing of DNA replication in vertebrates

Cdc6 is an essential component of the pre-replicative complex (pre-RC), the molecular machine that creates an unwound region of DNA on chromosomes and loads the replicative helicase thus initiating DNA replication in S-phase. Without Cdc6, DNA replication cannot start. In mammals, Cdc6 is a target of the APC and is ubiquitinated and degraded during G0 and G1 when APC activity is high. Cdc6 has Cdk1 consensus phosphorylation sequences embedded within, and around its D-box. Phosphorylation of these S/T*–P motifs by Cyclin-E/Cdk2 prevents recognition of the D-box and thereby stabilizes Cdc6. This phospho-inhibited degron allows accumulation of Cdc6 only during the short period of Cyclin-E/Cdk2 activation immediately prior to, and during early S-phase. The regulated destruction of Cdc6 may provide a mechanism to robustly prevent DNA replication during the extended G0 and G1 phases of differentiated vertebrate cells, thereby improving genome stability. Indeed, the structure of the D-box/Cdk module is conserved from frogs to humans [30].

11. Phosphorylation of the D-box of securin creates a positive feedback at anaphase onset

In addition to Chk1 consensus sites, securin also has Cdk1 consensus sites surrounding its D-box. As in the previous examples, phosphorylation of these sites by Cdk1 also disrupts recognition of securin by the APC thereby reducing the rate of securin ubiquitination by more than an order of magnitude. High levels of APC activity must accumulate prior to the initiation destruction of this recalcitrant phosphorylated securin. However, once some securin is destroyed, the release of separase leads to the activation of a phosphatase that efficiently dephosphorylates securin. This constitutes a positive feedback loop that leads to a more synchronous and decisive anaphase onset. Once again, at the heart of this positive feedback loop is the module consisting of a destruction motif that is negatively modulated by phosphorylation [31].

12. Conclusions

Protein stability is coupled to the phosphorylation state of proteins by the two modules of opposite logic described above: the phosphodegron that is activated by phosphorylation, and the phospho-inhibited degron that is inactivated by phosphorylation. The coupling of phosphorylation and ubiquitination networks provides opportunities for signal integration and the tight control of critical cell cycle transitions. The examples presented above merely scratch the surface; there is much to be discovered in this field, but the principles described so far provide a useful guide for future research.

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