Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control

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Cyclins and cyclin-dependent kinases (Cdks) are universal regulators of cell cycle progression in eukaryotic cells. Cdk activity is controlled by phosphorylation at three conserved sites, and many of the enzymes that act on these sites have now been identified. Although the biochemistry of Cdk phosphorylation is relatively well understood, the regulatory roles of such phosphorylation are, in many cases, obscure. Recent studies have uncovered new and unexpected potential roles, and prompted re-examination of previously assumed roles, of Cdk phosphorylation.

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Abbreviations

BIME	blocked in mitosis E
Cdk	cyclin-dependent kinase
KAP	Cdk-associated phosphatase
NIMA	never in mitosis A
PP2A	protein phosphatase 2A

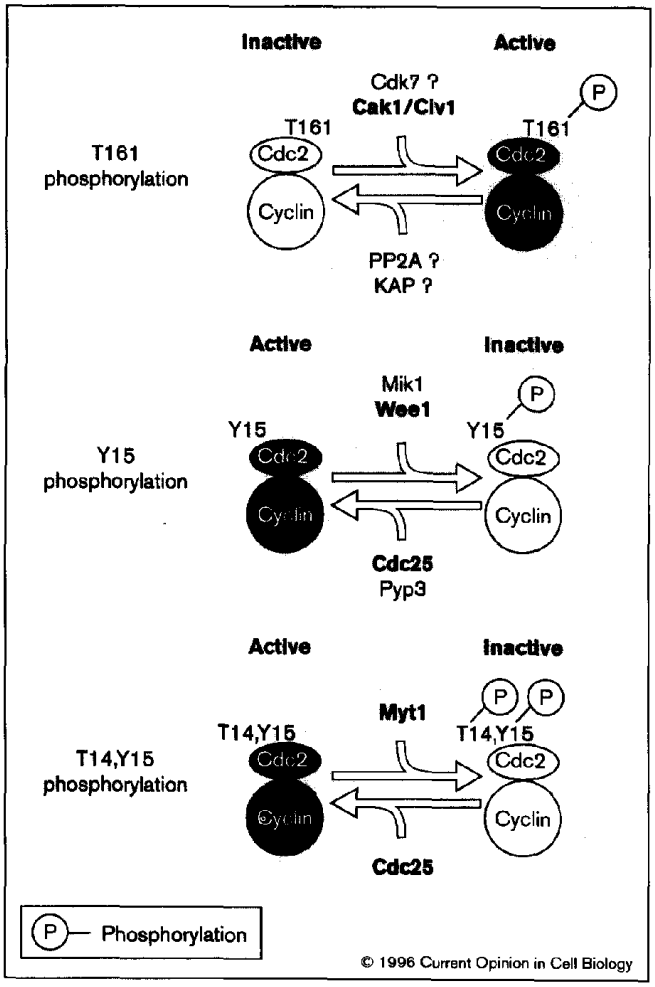
Introduction

Cyclin-dependent kinase (Cdk) activity requires binding of the Cdk to a cyclin and phosphorylation of the Cdk at a conserved threonine residue within the 'T-loop' (residue Thr161, or T161, in human Cdc2; see Fig. 1) [1]. Active, T161-phosphorylated and cyclin-bound Cdk can be inhibited by phosphorylation of two conserved residues within the catalytic cleft (residues Tyr15 [Y15] and Thr14 [T14] in human Cdc2; see Fig. 1). Although these regulatory phosphorylations were identified on, and are best characterized for, Cdc2 (the Cdk that promotes entry into mitosis; also called Cdc28 in budding yeast), similar pathways control the activity of other Cdks. Phosphorylation of chicken Cdc2 at S277 has also been described [2], but it is not clear how this affects its activity, or whether it applies to other Cdks. This review will focus on regulation at T161, Y15, and T14 of Cdc2/Cdc28 (the exact position of these residues varies between species, but in this review the same numbering will be used throughout for consistency).

T161 phosphorylation: in search of regulation

Biochemical purification approaches were successful in identifying an abundant kinase that phosphorylates T161. Surprisingly, this kinase is itself a Cdk–cyclin complex (namely Cdk7–cyclin H) that is present in the transcription factor TFIIH [3]. Apart from some reduction in activity in quiescent cells, there is no evidence for variation in Cdk7 activity during the cell cycle. *In vivo* confirmation that Cdk7 is indeed a relevant T161 kinase is currently lacking, however, and studies in budding yeast demonstrated that Kin28 (the closest relative of Cdk7 in this organism; also found in TFIIH) is not a T161 kinase *in vivo* or *in vitro* [4•]. A budding yeast T161 kinase has now been identified (namely Cak1/Civ1, a 43 kDa monomeric and rather unusual kinase [5•,6•]), and it remains to be seen

Figure 1



Regulatory phosphorylation of Cdc2. Phosphorylation at T161 (single-letter code for amino acids) is necessary for Cdc2 activity, whereas phosphorylation at either T14 or Y15 (single-letter code for amino acids) inhibits activity. Kinases and phosphatases that act on these sites are indicated. The major regulatory enzymes are indicated in bold type. In some cases, indicated by question marks, it is not known whether these enzymes act on Cdc2 *in vivo*. Shaded circles and ovals represent the active forms of the proteins, and open circles and ovals represent the inactive forms.

whether Cdk7 or a Cak1 homolog is responsible for T161 phosphorylation in other organisms. T161 dephosphorylation can be catalyzed by protein phosphatase 2A (PP2A) or a newly identified phosphatase, Cdk-associated phosphatase (KAP), *in vitro* [3,7]. Once again, it is unclear whether these or other phosphatases are responsible for T161 dephosphorylation *in vivo*. Given the uncertainty regarding the enzymes that control T161 phosphorylation, we cannot yet address the question of whether this modification is ever employed in a cell cycle regulatory capacity.

Size control in fission yeast utilizes Y15 phosphorylation

Genetic approaches in fission yeast have led to the identification of the enzymes responsible for the control of Cdc2 Y15 phosphorylation ([8–11]; reviewed in [12,13]). The kinases Wee1 and Mik1 phosphorylate Y15, whereas the phosphatases Cdc25 and Pyp3 dephosphorylate Y15 (Fig. 1). Wee1 and Cdc25 are primarily responsible for regulation of Y15 phosphorylation, whereas Mik1 and Pyp3 are minor partners whose role becomes apparent only when the major partners are compromised.

In fission yeast, a G_2-phase size control operates under rapid growth conditions, such that the onset of mitosis is dependent on reaching a critical cell size. The size at mitosis was shown to be dependent on *wee1* and *cdc25* gene dosage, so that as little as a threefold increase in gene dosage has significant effects on the timing of mitosis (Wee1 delays mitosis, whereas Cdc25 advances mitosis) [8,9]. These observations demonstrated that the timing of entry into mitosis was controlled by the *cdc25:wee1* ratio. As cells grow during G_2 phase, the levels of *cdc25* mRNA and protein increase until mitosis is triggered, after which there is a sharp drop in both mRNA and protein abundance [14]. It has been proposed that this accumulation pattern results in a gradually increasing *cdc25:wee1* ratio during G_2 phase, and that the ratio becomes sufficient to trigger mitosis when cells reach the critical size. Although this model is attractive, proof will require determination of Wee1 levels (and activity) through the cell cycle. Wee1 appears to be a labile protein and, to date, it has not been technically feasible to accurately measure endogenous levels of *wee1* expression.

Recent studies have begun to address the basis for the oscillation in Cdc25 levels through the cell cycle. Cdc25 accumulates to high levels in *cdc* mutants that are blocked for cell cycle progression, whether the block is in G_1, S, or G_2 phase [15••]. This suggests that Cdc25 accumulation during interphase depends on the total time spent in interphase, rather than the exact position in the cell cycle. Based on this, Kovelman and Russell [15••] propose that Cdc25 periodicity arises due to the rapid elimination of *cdc25* mRNA and protein that occurs during mitosis, followed by a constant rate of synthesis during interphase. In this model, size control is, in essence, a timing mechanism: mitosis is initiated when *de novo* accumulation of Cdc25 surpasses the threshold set by the (presumed) constant Wee1 activity, yielding activation of Cdc2. A further twist in this model is provided by the observation that the extent of phosphorylation and the specific activity of Cdc25 are elevated in cells blocked in S phase compared with cells blocked in G_1 phase [15••]. Thus, as Cdc25 accumulates in the unperturbed cell cycle it may also be increasing in specific activity.

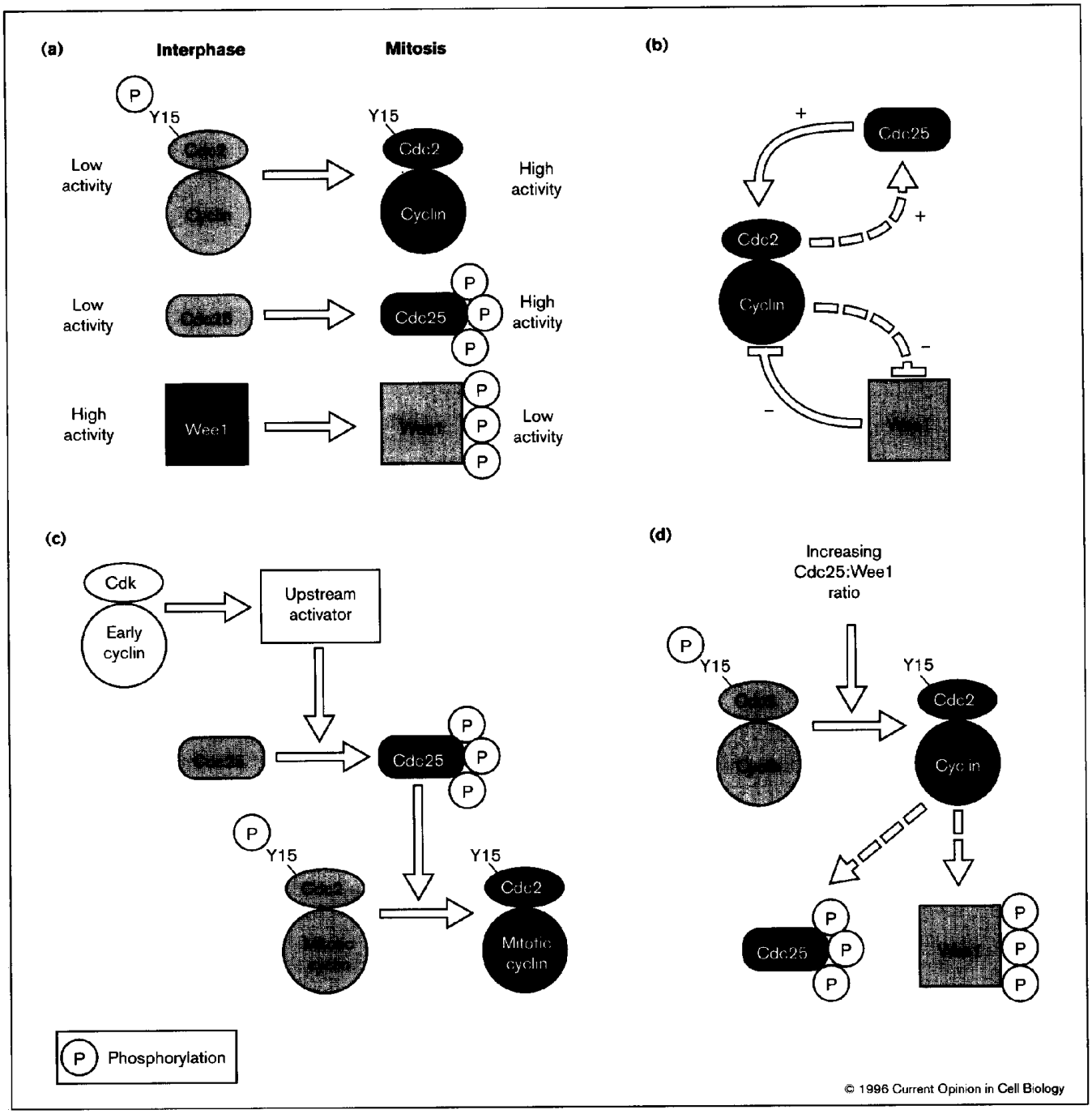
The mechanism behind the mitotic elimination of Cdc25 remains mysterious: it is currently unclear whether destabilization of the mRNA or protein (or both) is primarily responsible. A potentially important clue was reported recently by Nefsky and Beach [16•], who identified a protein called Pub1 that is required for efficient Cdc25 ubiquitination. Pub1 contains a domain homologous to a domain of E6-AP, a protein–ubiquitin ligase that targets p53 for degradation in human papilloma virus infected cells [17]. Like E6-AP, Pub1 forms an ubiquitin thioester that is presumed to be an intermediate in target protein ubiquitination, and Nefsky and Beach [16•] propose that Pub1 directly ubiquitinates Cdc25 and targets it for destruction. It remains to be determined whether Cdc25 stability varies through the cell cycle, and if so whether Pub1 is responsible for that regulation.

Feedback loops for entry into mitosis

Studies on the Cdc25 proteins from *Xenopus*, humans, and fission yeast have shown that Cdc25 is hyperphosphorylated during mitosis relative to during interphase, and that this leads to a significant enhancement of its phosphatase activity (reviewed by Dunphy [13]; Fig. 2a). Similar studies on *Xenopus* and human Wee1 have also uncovered mitotic hyperphosphorylation, in this case leading to an inhibition of its kinase activity [13,18•,19,20] (Fig. 2a). Cdc2 may itself contribute to these hyperphosphorylations, but other kinases must also play a major role [19,21,22]. Recently, a Polo-like kinase (Plx1 [23]) that binds to and hyperphosphorylates Cdc25 was purified from *Xenopus*. Plx1-induced phosphorylation of Cdc25 occurs at multiple sites and generates epitopes on Cdc25 that are recognized by the mpm2 monoclonal antibody. Mpm2-binding phosphoepitopes are generated during mitosis on a number of proteins, including not only Cdc25 but also Wee1, suggesting that Plx1 may also contribute to Wee1 hyperphosphorylation in mitosis [23].

These observations reveal the potential for positive feedback loops contributing to Cdc2 activation at the $G_2 \rightarrow M$ transition (Fig. 2b). Such post-translational feedback control can explain early observations regarding mitosis promoting factor autoamplification [24], and is attractive in that it provides a mechanism by which to sharpen the transition into mitosis. For these reasons, positive feedback models have been popular and sometimes taken for granted in the cell cycle control field.

Figure 2



Models for the relationship between activation of Cdc2 and phosphorylation of its regulators (Wee1 and Cdc25) at the $G_2 \rightarrow M$ transition. Darkly shaded shapes represent high activity of the protein, and lightly shaded shapes represent low activity of the protein. **(a)** Cdc2–cyclin complexes are inhibited through Cdc2 Tyr(Y)15 phosphorylation during interphase, and activated (so Y15 is not phosphorylated) during mitosis. Both Cdc25 (which dephosphorylates and activates Cdc2) and Wee1 (which phosphorylates and inhibits Cdc2) become hyperphosphorylated in mitosis, leading to activation of Cdc25 and repression of Wee1. **(b)** A positive feedback model proposes that, in the transition from interphase to mitosis, activation of a small amount of Cdc2 rapidly causes hyperphosphorylation of some Cdc25 and Wee1, leading to further Cdc2 activation and so on until all of the Cdc2 is active (all Y15s are dephosphorylated) and all of the Cdc25 and Wee1 proteins are hyperphosphorylated. The dashed arrows indicate that the path from Cdc2 activity to Cdc25 and Wee1 hyperphosphorylation involves intermediary steps, likely to include Polo-like kinases (see text). **(c)** An alternative model for Cdc2 activation, in which Cdc25 hyperphosphorylation is triggered by upstream regulators (likely to include both a Polo-like kinase and Cdk–cyclin complexes); activated Cdc25 then dephosphorylates and activates Cdc2. A similar model could be drawn for Wee1. **(d)** In this model, the interphase activity of Cdc25 is sufficient to cause full Cdc2 activation in the absence of Cdc25 and Wee1 hyperphosphorylation (in fission yeast, this may occur when sufficient Cdc25 protein accumulates to overcome the Wee1 present). Hyperphosphorylation of Cdc25 and Wee1 occurs downstream of Cdc2 activation and serves to 'lock' Cdc2 in the dephosphorylated state.

Although the potential for positive feedback in Cdc2 activation has been clearly established, the relevance of positive feedback as a physiological mechanism is much harder to prove. In this context, it is worth noting that a potential positive feedback loop at the $G_1 \rightarrow S$ transition was recently found to be irrelevant to the cell cycle in budding yeast. In that case, it had been established that transcription of the G_1-phase cyclin genes *CLN1* and *CLN2* could be stimulated by the Cln1–Cdc28 and Cln2–Cdc28 kinase complexes [25,26]. As *CLN1* and *CLN2* transcripts accumulate from very low levels to maximal levels within a relatively short interval in late G_1 phase [27], it seemed likely that as Cln1 and Cln2 proteins accumulated they stimulated further *CLN1* and *CLN2* transcription to bring about a rapid $G_1 \rightarrow S$ transition. Subsequent experiments showed, however, that maximal induction of *CLN1* and *CLN2* transcripts occurs with normal kinetics regardless of whether Cln1 and Cln2 are present: transcriptional induction of *CLN1* and *CLN2* is solely dependent on a divergent cyclin, Cln3 [28••,29••]. Furthermore, the activation of Cln1–Cdc28 and Cln2–Cdc28 triggers other cell cycle processes that culminate in repression of *CLN1* and *CLN2* transcription [28••,30]. Thus, the potential for positive feedback was never realized in this system.

This example from budding yeast serves to focus attention on a possible scenario for $G_2 \rightarrow M$ control in which positive feedback does not play a role (Fig. 2c). In this model, some upstream regulator (Plx1 would be a good candidate) brings about full activation of Cdc25 (and/or inactivation of Wee1) with no requirement for cyclin B–Cdc2 activity (and hence no positive feedback). Cdc2 activation ensues, triggering entry into mitosis, and perhaps Cdc25 destruction. The only data that appear to contradict this model come from the observation that, in fission yeast, Cdc2 is required for Cdc25 activation [15••]. This could be simply accommodated by postulating that, as in budding yeast, an upstream cyclin–Cdk complex contributes to activation of the upstream regulator (Fig. 2c). This model would fit well with recent data from Guadagno and Newport [31••] showing that Cdk2 activity (probably in a complex with cyclin E) is required for both Cdc25 hyperphosphorylation and Cdc2 activation in cycling extracts from *Xenopus* eggs. This requirement was evident even in cytoplasmic extracts lacking nuclei, suggesting that it was unrelated to the previously characterized Cdk2 requirement for DNA replication.

Other scenarios can also be envisaged. For instance, it may be that the basal interphase activity of Cdc25 is sufficient to provoke full Cdc2 activation, leading to subsequent hyperphosphorylation of Cdc25 (Fig. 2d). In this model, Cdc25 activation in mitosis would simply constitute an insurance mechanism to ensure that Cdc2 remains active until the cyclin is degraded. Distinguishing between the different models will require examination of the effects of preventing Cdc25 or Wee1 hyperphosphorylation (without perturbing basal activity) *in vivo*.

Developmental regulation of the cell cycle

Elegant studies in *Drosophila* embryos have shown that different cell cycle regulators become rate-limiting for cell cycle progression at various stages of development (reviewed by Edgar [32••]). In particular, transcription of *cdc25* (called *string* in *Drosophila*) is rate-limiting for the timing of many postblastoderm mitoses, and the correct pattern of *cdc25* transcription is therefore critical for morphogenesis during development. *cdc25* has a large (>40 kb) promoter region with multiple elements that respond locally to various pattern formation genes [33]. This suggests that the positional information embodied in the complex expression patterns of these genes is translated into a particular spatiotemporal pattern of cell division through regulation of *cdc25* transcription.

Checkpoint control: DNA replication

Seven years ago, Enoch and Nurse [34] reported that certain mutations affecting Cdc2 Y15 phosphorylation in fission yeast could override the checkpoint control that delays entry into mitosis in response to incomplete DNA replication. This result was the first to implicate Cdc2 as a target of checkpoint controls, and suggested that the DNA-replication checkpoint might inhibit entry into mitosis simply by tilting the balance of Cdc25 and Wee1 activities in favor of Cdc2 phosphorylation. However, cells lacking both Wee1 and Cdc25 maintained the ability to respond to incomplete DNA replication by delaying entry into mitosis, showing that neither Wee1 nor Cdc25 could be the sole target of the checkpoint control [35]. Thus, if Cdc2 Y15 phosphorylation is regulated by the checkpoint, there must be several regulatory inputs affecting both the major and the minor enzymes that act on Cdc2 Y15.

Cdc2 Y15 phosphorylation is not always required for checkpoint function

In the past few years, considerable effort has been devoted to studies of the mechanisms underlying checkpoint arrest of the cell cycle. One conclusion from these studies is that the requirement for an intact Cdc2 Y15 phosphorylation pathway varies from species to species. In fission yeast, the ability to phosphorylate Cdc2 Y15 is absolutely required for inhibition of mitosis in cells that have not completed DNA replication [10,34]. In contrast, recent studies in *Xenopus* egg extracts [36•], human cells [37•], and *Aspergillus nidulans* [38•], combined with earlier studies in budding yeast [39,40], suggest that nonphosphorylatable Cdc2 or Cdc28 mutants cannot override a checkpoint arrest induced by drugs that prevent DNA replication. These studies establish that there must exist some non-Y15-related mechanism(s) whereby the checkpoint can prevent entry into mitosis.

Intriguingly, the ability to phosphorylate Cdc2 Y15 was critical for the checkpoint-induced delay of mitosis in *A. nidulans* cells exposed to low levels of drugs sufficient

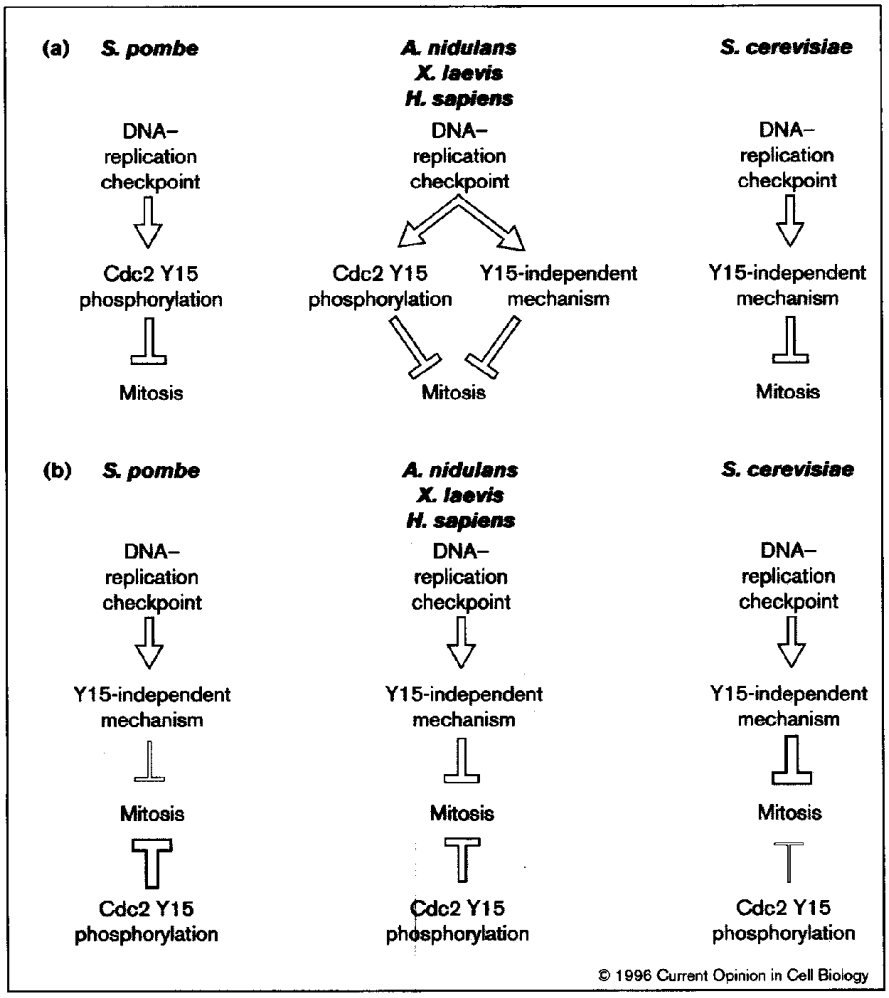
to slow, but not block, DNA replication [38•]. In addition, human cells expressing the nonphosphorylatable Cdc2 mutant displayed a significant loss of viability following addition of the drug, suggesting that the checkpoint was at least partially compromised (C McGowan, personal communication). In these cases, it appears that the ability to phosphorylate Y15 is critical for checkpoint operation when DNA replication is merely delayed, but is perhaps dispensable when DNA replication is completely blocked. These data have been interpreted [38•] as evidence for two distinct checkpoint pathways responding to incomplete DNA replication: one pathway which has a sensitive detection threshold and requires Cdc2 Y15 phosphorylation; and another which is relatively insensitive (hence large amounts of drugs are required to trigger this pathway) and which operates independently of Y15 phosphorylation (Fig. 3a). The species specificity then arises because different pathways are prevalent in different cells: fission yeast use only the Y15-dependent pathway, *Aspergillus* and human cells use both, and budding yeast use only the Y15-independent pathway.

Although this model accounts for all of the observations, we believe that an alternative and perhaps simpler model is also consistent with the available data (Fig. 3b; a similar model has been discussed by Kumagai and Dunphy [36•]). In this model, the checkpoint operates exclusively through the Y15-independent pathway(s) in all organisms. This pathway responds in a graded fashion to the checkpoint stimulus (incomplete DNA replication), and this graded signal leads to inhibition of Cdc2 through a mechanism that does not involve regulation of Y15 phosphorylation.

The species specificity in this model arises due to the extent to which Cdc2 Y15 phosphorylation is prevalent in the unperturbed cycle (Fig. 3b). A G_2-phase delay introduced by a Y15-independent checkpoint mechanism would allow Y15-phosphorylated Cdc2 to accumulate due to the continued action of constitutively active Wee1-like kinases. This Y15 phosphorylation would be expected to assist in the suppression of Cdc2 activity, and the degree of assistance provided would depend on the prevalence of Cdc2 Y15 phosphorylation in different cells. In fission

Figure 3

Two models for the operation of the DNA-replication checkpoint in different species. **(a)** There are two separate checkpoint mechanisms, one of which modulates the enzymes that control Tyr(Y)15 phosphorylation and the other of which does not. Fission yeast use the first mechanism (and hence require Cdc2 Y15 phosphorylation for checkpoint function), whereas budding yeast use the second (and hence have a functional checkpoint even in the absence of Cdc28 Y15 phosphorylation). Other organisms have a mixed checkpoint that uses both pathways. **(b)** All cells respond to incomplete DNA replication through a pathway that does not affect the enzymes that control Cdc2 Y15 phosphorylation (i.e. through a Y15-independent mechanism). In addition, Y15 phosphorylation occurring normally as part of the cell cycle assists the checkpoint in preventing entry into mitosis. The prevalence of Y15 phosphorylation varies between species (indicated by the thickness of the arrows) and therefore provides different degrees of assistance to the checkpoint.



yeast, Y15 phosphorylation is thought to occur at high stoichiometry on Cdc2–Cdc13 complexes during interphase, and cells that cannot phosphorylate Cdc2 Y15 undergo an extreme and catastrophic acceleration of mitosis [10]. In budding yeast, however, Y15 phosphorylation is thought to occur at low stoichiometry (if at all) on Cdc28–Clb complexes, and cells that cannot phosphorylate Cdc28 Y15 do not exhibit any change in the kinetics of mitosis [39–42]. In *Aspergillus* and human cells, an intermediate situation may exist, wherein cells expressing the Cdc2 nonphosphorylatable mutant undergo a mild acceleration of mitosis [37•,38•].

These species-specific differences suggest that checkpoint pathways in different organisms may have evolved in environments with differing levels of Cdc2 Y15 phosphorylation. In fission yeast, the prevalence of Y15 phosphorylation might allow a checkpoint pathway to prevent entry into mitosis via inhibitors that would be insufficient or ineffective when Y15 is dephosphorylated. In contrast, a checkpoint-induced inhibitor in budding yeast would have to operate in the absence of significant Y15 phosphorylation. Therefore, the differing ability of nonphosphorylatable Cdc2 mutants to override a checkpoint arrest in different species could be explained by the degree to which tyrosine phosphorylation would be expected to assist in maintaining the checkpoint arrest in these species. In the simplest case, a single ancestral checkpoint pathway may have adapted to produce different 'strengths' of inhibition in the different organisms (Fig. 3b).

Regulation of Cdc2 Y15 phosphorylation by the checkpoint

This model focuses attention on the question of whether or not there is ever a situation in which the DNA-replication checkpoint modulates the enzymes that control Y15 phosphorylation. This question has been addressed in *Xenopus* egg extracts, with apparently contradictory results. In one study, Smythe and Newport [43] found that Wee1-like activities in checkpoint-arrested extracts were elevated fivefold to tenfold compared with those in cycling extracts. In contrast, Kumagai and Dunphy [36•] found no difference in such activities between checkpoint-arrested and nonarrested extracts. The assay in both cases was initiated by adding tagged recombinant cyclin proteins to different extracts. These added cyclins formed complexes with the free Cdc2 in the extracts and therefore created fresh substrate for Wee1-like kinases. The initial rate of phosphorylation of this tagged cyclin–Cdc2 substrate by Wee1-like kinases was then used to estimate the total Cdc2 Y15-directed tyrosine kinase activity in the extracts.

One difficulty in this kind of assay is that the tagged substrate becomes fully phosphorylated within a short time. Kumagai and Dunphy [36•] showed full phosphorylation of the substrate at five minutes, whereas Smythe and Newport's substrates [43] still appeared to be in the initial phase of phosphorylation at ten minutes. This is probably explained by the fact that Smythe and Newport added much more tagged cyclin (2–3 μM final concentration) than Kumagai and Dunphy (30–40 nM final concentration). As their substrate was phosphorylated so rapidly, it may have been difficult for Kumagai and Dunphy [36•] to detect an enhancement of the initial rate of phosphorylation. Another experimental difference was that Smythe and Newport [43] used cycling extracts with ongoing protein (and cyclin) synthesis, whereas Kumagai and Dunphy [36•] employed interphase extracts containing cycloheximide. Conceivably, a checkpoint-mediated mechanism inducing Wee1-like kinases might decay in the absence of protein synthesis, and would therefore be missed in the Kumagai and Dunphy protocol. The cycloheximide-containing extracts did exhibit a robust checkpoint, however, so protein synthesis is not essential for all checkpoint function, and would have to be required specifically for the Y15-regulatory pathway.

These considerations suggest the possibility that a real checkpoint-induction of Wee1-like kinases may have been obscured in the experiments of Kumagai and Dunphy [36•]. It is also possible, however, that they are correct and that there is no induction of Wee1-like kinases by the DNA-replication checkpoint. Conceivably, a misleading apparent induction of Wee1-like kinases in the experiments of Smythe and Newport [43] may have resulted from the Y15-independent checkpoint mechanism proposed by Kumagai and Dunphy [36•]. In one scenario, the large amount of cyclin added by Smythe and Newport may have led to a highly active cyclin–Cdc2 complex in the extract during the 10-minute assay. As mentioned above, mitotic hyperphosphorylation is known to repress Wee1 activity. Thus, the bolus of active Cdc2 generated at the beginning of the assay may have had an inhibitory effect on Wee1-like kinases in the extract. In the checkpoint-arrested extract, the Y15-independent inhibitory pathway may have prevented (or slowed) the activation of Cdc2 by added cyclin, so that an uninhibited Wee1-like activity would be maintained during the short assay time interval. Compared with the artificially repressed activity in the nonarrested extract, this might falsely appear to be a checkpoint stimulation of Wee1-like kinases.

Another experimental difference between the two studies was that Smythe and Newport [43] used a GST (glutathione-S-transferase)-tagged sea urchin cyclin produced in bacteria, whereas Kumagai and Dunphy [36•] used a His-tagged human cyclin produced in insect cells. A puzzling property of the sea urchin cyclin was that its addition to checkpoint-arrested extracts promoted override of the checkpoint and entry into mitosis. Had the sea urchin cyclin been the one that failed to reveal any difference between checkpoint-arrested and nonarrested extracts, we might with some confidence have ascribed that result to an aberrant property of this particular cyclin.

However, it is much harder to explain the finding that the use of this cyclin did in fact reveal a significant checkpoint induction of Wee1-like kinases.

The question of whether Wee1-like kinases are induced by the DNA-replication checkpoint was also addressed in human cells by McGowan and Russell [18•]. They found that the activity of the major assayable Wee1-like kinase in soluble HeLa cell lysates was identical whether the lysates were prepared from cycling or checkpoint-arrested cells. Thus, at least this kinase did not appear to be induced by the checkpoint.

In addition to measuring the effect of the checkpoint on Wee1-like kinases in the extract, Kumagai and Dunphy [36•] attempted to measure checkpoint effects on the activity of Cdc25-like phosphatases. They observed no difference between checkpoint-arrested and cycling extracts, and concluded that regulation of Cdc25-like phosphatases by the checkpoint was unlikely. However, the activity in their assay was very low, and a checkpoint-mediated inhibition of Cdc25-like factors may have been undetectable. In summary, the available data do not permit a definitive conclusion as to whether Cdc2 Y15 directed enzymes are regulatory targets for the DNA-replication checkpoint.

If regulation of Cdc2 Y15 phosphorylation does not mediate the checkpoint-induced delay in mitosis, how does the checkpoint work? An elegant experiment by Kumagai and Dunphy [36•] suggested that a titratable Cdc2 inhibitor might play a role in *Xenopus* extracts. They first showed that checkpoint-arrested extracts could inhibit added cyclin–Cdc2 complexes that were sufficient to induce mitosis in the absence of a checkpoint, even if the added Cdc2 was not phosphorylatable at Y15 (or T14). This demonstrated the existence of a Y15-independent checkpoint pathway (see above). They then showed that the checkpoint arrest could be overridden by adding sufficient quantities of cyclin–Cdc2 complexes containing a catalytically inert mutant of Cdc2. This suggested that a titratable inhibitor in the extracts may have bound to the added (inactive) complexes, releasing the endogenous complexes to promote entry into mitosis. Recently, a large membrane-associated Cdc2 inhibitor was described in *Xenopus* egg extracts [44•], but it is not known whether this inhibitor is induced by the checkpoint.

In *Aspergillus*, the BIME (blocked in mitosis E) protein is required for an effective checkpoint [38•,45]. One role for BIME is apparently to suppress the activity of the protein kinase NIMA (never in mitosis A) [38•], which is essential for entry into mitosis in *Aspergillus*. Whether this constitutes the relevant (or only) checkpoint-related role of BIME remains to be determined. Although these observations provide tantalizing clues, we do not yet have a clear picture of how this checkpoint mediates cell cycle arrest.

Checkpoint control: DNA damage

Carr and colleagues [46,47] have argued that, unlike the DNA-replication checkpoint, the checkpoint responding to DNA damage does not require Y15 phosphorylation. This conclusion was based on experiments showing that fission yeast strains (including *wee1* mutants and *cdc2* mutants) that were unable to sustain a DNA-replication checkpoint could, nevertheless, delay mitosis in response to DNA damage. These strains were partially impaired in their ability to inhibit Cdc2 through Y15 phosphorylation, suggesting that the DNA-damage checkpoint could delay mitosis even in the absence of such phosphorylation. However, N Rhind and P Russell (personal communication) recently found that in *wee1 mik1* double mutants (which are completely unable to phosphorylate Y15), the DNA damage checkpoint response is severely impaired. This demonstrates that Cdc2 Y15 phosphorylation is required for the DNA-damage checkpoint to operate in fission yeast, although the requirement appears to be less stringent than for the DNA-replication checkpoint. In human cells, Y15 phosphorylation is important for the delay of mitosis by the DNA-damage checkpoint, but some delay still occurs in cells containing the nonphosphorylatable Cdc2 mutant [37•]. In budding yeast, the DNA-damage checkpoint does not require Cdc28 Y15 phosphorylation [39–42]. Thus, a species-specific pattern of Y15-phosphorylation requirements applies to the DNA-damage checkpoint as well as to the DNA-replication checkpoint. The key question of whether the DNA-damage checkpoint causes regulation of Wee1- or Cdc25-like activities has not yet been addressed.

Checkpoint control: morphogenesis

In budding yeast, a nonphosphorylatable Cdc28 mutant does not appreciably affect cell cycle kinetics, and does not affect the ability of the DNA-damage and DNA-replication checkpoints to delay cell cycle progression [39–42]. It seems likely that Cdc28 is not phosphorylated to a significant stoichiometry during the unperturbed cell cycle in this organism. However, Cdc28 tyrosine phosphorylation is detectable in cells arrested in S phase [39], and Wee1 [41] and Cdc25 [48] homologs have been identified in budding yeast. These findings raised the question of whether the Y15-phosphorylation pathway in this organism is vestigial or whether it functions in some other physiological setting.

It has been known for some time that changes in the growth environment (e.g. shifts in temperature [49] or osmolarity [50]) of budding yeast provoke a stress response that involves a transient depolarization of the actin cytoskeleton, leading to delays in bud formation. Despite these delays, the cell cycle in stressed cells remains well coordinated with bud formation. Recently, a morphogenesis checkpoint was described in budding yeast that delays nuclear division in cells that have failed to form a suitable bud [51••]. It was proposed that this checkpoint

is responsible for adjusting the cell cycle of stressed cells to prevent the formation of binucleate cells.

Strikingly, Cdc28 Y15 phosphorylation was found to be essential for the ability of the morphogenesis checkpoint to delay nuclear division [51••]. Indeed, the timing of nuclear division in cells with depolarized actin was found to be very sensitive to the gene dosage of the *wee1* and *cdc25* homologs [52]. Thus, as in fission yeast, the cell cycle in budding yeast cells with depolarized actin displays a long G_2 phase, and the timing of nuclear division is determined by the *cdc25:wee1* ratio. To explain these observations, it was proposed that the morphogenesis checkpoint regulated the Wee1 and/or Cdc25 homologs in response to defects in bud formation [52]. As for the other checkpoints, however, direct regulation of these activities remains to be demonstrated.

T14 phosphorylation: Y15 assistant?

Phosphorylation of Cdc2 T14 has been observed in metazoans [2,53,54]. Recently, T14 phosphorylation was also detected in fission yeast [55], but it is unclear whether it ever occurs at high enough stoichiometry to serve a cell cycle regulatory role in this organism. Mutation of *cdc2* so that the protein cannot be phosphorylated at T14 does not cause any cell-cycle or checkpoint perturbation in any system analyzed thus far. However, double mutants that cannot be phosphorylated at either T14 or Y15 often result in an enhanced defect compared with defects produced by mutations at Y15 alone [53,56]. These findings suggest that T14 and Y15 phosphorylation performs similar roles, yielding a double block to Cdc2 activation. One reason for employing a double block may be that the most abundant protein phosphatases in the cell are specific for either serine/threonine- or tyrosine-phosphorylated proteins. The probability that such phosphatases might accidentally (and perhaps catastrophically) activate Cdc2 is greatly reduced by the requirement that both T14 and Y15 be dephosphorylated. Thus, activation of Cdc2 would depend uniquely on the highly specific dual-specificity threonine/tyrosine phosphatase, Cdc25.

Although Cdc25 is able to dephosphorylate both T14 and Y15 [57,58], Wee1 is only able to phosphorylate Y15 of Cdc2 [59,60]. A dual-specificity kinase capable of promoting phosphorylation of both T14 and Y15 was found to be associated with membranes in *Xenopus* egg extracts [61,62], and is almost certainly the same as the kinase Myt1 which was identified in a PCR search for Wee1 homologs in *Xenopus* [63•]. Like Wee1, Myt1 is hyperphosphorylated and inhibited during mitosis [63•].

Myt1 contains a putative transmembrane domain [63•], and a human Myt1 homolog resides primarily in the endoplasmic reticulum (H Piwnica-Worms, personal communication). In contrast, Wee1 resides in the nucleus, at least when overexpressed [18•]. This suggests that the two kinases may be phosphorylating different populations of Cdc2. This idea is particularly attractive in light of the finding that different cyclin B isoforms (that presumably form the Cdc2 complexes that are targets for regulation by Wee1 and Myt1) display distinct subcellular localizations in human cells [64••].

Conclusions

The past year or two have produced advances on many fronts in the field of Cdk regulation. Novel kinases that phosphorylate Cdc2 at T161 (Cak1/Civ1) and T14/Y15 (Myt1) have been identified and provide new avenues along which to study Cdc2 regulation. Cdc2 phosphorylation independent mechanisms have been discovered to play a key role in the DNA-replication checkpoint in many, if not all, cells. A morphogenesis checkpoint may explain the elusive nature of Cdc28 Y15 phosphorylation in budding yeast, and new pieces have been added to the story of size control in fission yeast.

Moving from the study of what can happen in laboratory assays to the study of what *does* happen in a physiological setting poses significant challenges for the coming years. As our understanding of cell cycle control increases, the subtlety and sophistication of experimental analyses are growing. Problems such as the basis of size control, the role of positive feedback loops in cell cycle control, and the mechanisms whereby checkpoints affect cell cycle progression are not easy to answer, but there is every reason to hope that the solutions will emerge from a concerted attack by the highly active cell cycle community.

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