Cell cycle: **Reaching for a role for the Cks proteins**

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The Cks proteins are essential components of the cyclin-dependent protein kinases that regulate mitosis in all eukaryotes, but their precise function remains obscure. The crystal structures of several Cks proteins offer insights into their roles during the cell cycle.

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One of the most tantalizing questions in the control of the cell cycle concerns the function of the Cks proteins. Why tantalizing? Tantalus was punished by being forever hungry and thirsty in a place where abundant fruit hung overhead and cool water lapped at his feet. Yet when he put out his hand, the fruit moved just out of reach, and when he bent down, the water receded. This can be compared to studying the Cks proteins: work on these proteins has been intensive since their identification 10 years ago, but what they do remains elusive. Furthermore, some experiments indicate that the Cks proteins are required for entry into mitosis, others implicate them only in exit from mitosis, and still others in the control of transitions between the G1 and S phases of the cycle and between the G2 and M phases. The recently solved crystal structures of the Cks proteins alone [1–4], and of the human CksHs1 as part of a binary complex with the cell-cycle regulatory kinase Cdk2 [5], suggest that Cks proteins can have two different conformations, one of which precludes binding to the kinase. The structures also give clues to the function of the Cks proteins in cell-cycle control.

The Cks family is composed of small proteins (9–18 kDa) that perform essential, conserved functions in the cell cycle. The first to be isolated was the product of *suc1* [6], which is a suppressor of defective alleles of *cdc2*, the gene encoding the primary cyclin-dependent kinase (Cdk) in fission yeast. Similarly, the homologous Cks in budding yeast was found by virtue of its interaction with the budding yeast Cdk (Cdc28), hence its designation as Cks1 (Cdc28 kinase subunit 1) [7]. The phenotypes of the two yeasts when *CKS1* or *suc1* is deleted or overexpressed provide contradictory indications of the protein's role. Budding yeast with a defective Cks1p [8] or fission yeast overexpressing Suc1 [9] arrest in G2 phase, whereas in the absence of Suc1 fission yeast enter mitosis but arrest there with high levels of Cdc2 kinase activity (see Fig. 1a) [10]. (Differences in phenotype between the budding yeast temperature-sensitive mutation and the fission yeast deletion strain might also be due to a threshold effect, because the store of a stable protein gradually runs out in a deletion strain, whereas it is inactivated at one point in time in a temperature-sensitive strain that is shifted to the restrictive temperature.) Despite these differences, the two yeast proteins have been shown by complementation studies to be true homologues. Similarly, two human homologues, CksHs1 and CksHs2, which were isolated using the polymerase chain reaction [11], can each functionally replace Cks1p in budding yeast.

A gene that suppresses a mutation in another gene often does so because their encoded proteins bind to one another. So it is for the Cks proteins and the cell-cycle regulatory kinases. Co-immunoprecipitation studies have shown that Cks proteins are bound to the mitotic cyclin-dependent kinase in yeast [12], human cells [13] and frog eggs [14]. This conserved interaction underlies the widespread use of Suc1 ($p13^{suc1}$) as an affinity chromatography matrix to purify cyclin–Cdk complexes from a variety of systems.

The Cks proteins have most often been implicated in modulating the tyrosine-phosphorylation state of the major mitotic Cdk, the cyclin B–Cdc2 complex. Before mitosis, cyclin B–Cdc2 is kept inactive by phosphorylation on Tyr 15 of the Cdc2 subunit. In cytoplasmic extracts of *Xenopus* eggs, adding an excess of the Cks protein Xe-p9 correlates with a delay in the tyrosine-dephosphorylation of the Cdc2 subunit [14]. Immunodepleting a frog-egg extract of Xe-p9 has different effects depending on the stage of the cell cycle. Thus, interphase extracts lacking Xe-p9 are unable to enter mitosis, and they maintain Cdc2 in its Tyr 15-phosphorylated state (as do extracts with excess Xe-p9), whereas mitotic extracts are unable to re-enter interphase, correlating with a defect in cyclin B destruction [14]. This recapitulates the phenotypes of Cks-deficient budding yeast (G2-phase arrest) and fission yeast (M-phase arrest) — although the G2-phase arrest in budding yeast is not likely to be due to a defect in Cdc28 tyrosine-dephosphorylation, because this is not required for the normal regulation of mitosis in the budding yeast. But under some conditions budding yeast with a defective Cks1p arrest in G2 phase without having budded during the preceding G1 phase, and the one process in the normal cell cycle in which Cdc28 tyrosine-phosphorylation has been shown to play a part is the coordination of DNA replication with budding [15]. Thus, there is considerable circumstantial evidence linking the Cks proteins with the regulation of Cdc2 tyrosine phosphorylation (Fig. 1b). Is this borne out by the crystal structures?

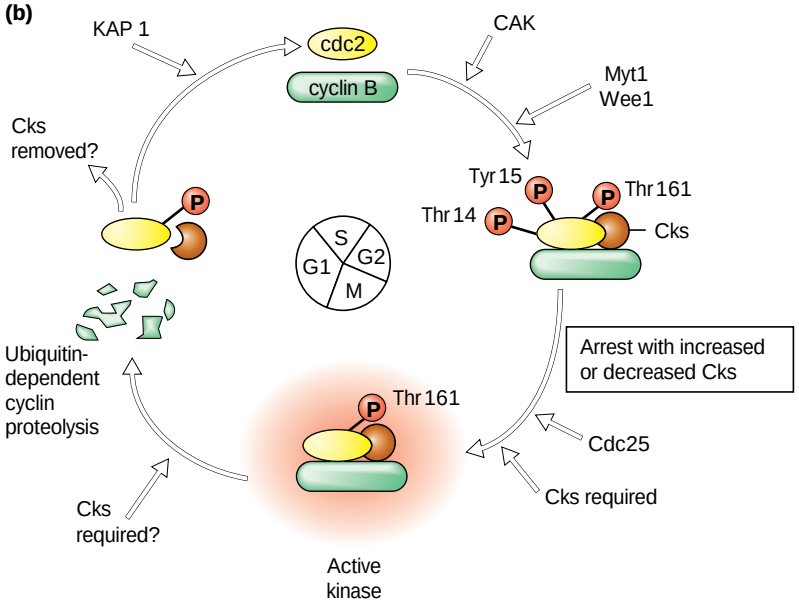
The crystal structures of fission yeast Suc1 [1,3,4] and human CksHs1 [16] and CksHs2 [2] have been solved.

Figure 1

(a)

	Cks deficiency	Cks excess
Budding yeast	G2 arrest	G2 delay
Fission yeast	M-phase arrest	G2 arrest
Frog egg extract	Interphse extract, G2 arrest, Mitotic extract, M-phase arrest	G2→M-phase transition delayed

(b)



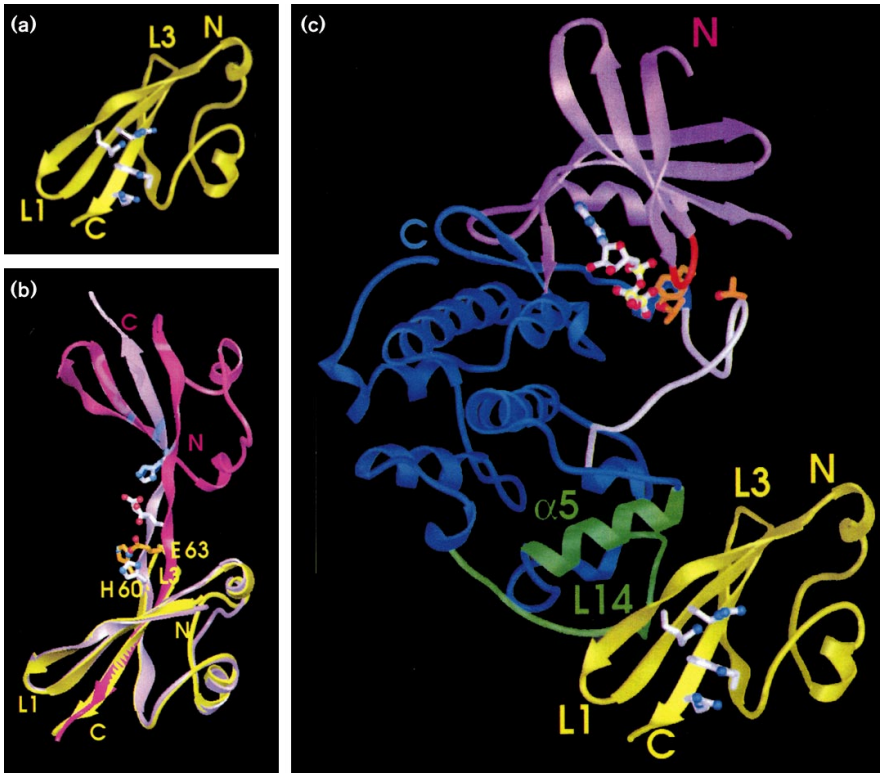
(a) Summary of the effects of Cks deficiency or excess in different organisms. (b) The possible points during the cell cycle when Cks is required (see text for details).

These proteins can be crystallized in either of two forms: as monomers or as strand-exchanged dimers (Fig. 2). Monomeric Cks proteins are composed of four anti-parallel β sheets and two α helices (Fig. 2a), and for CksHs1 the carboxy-terminal four-stranded β sheet bears a remarkable resemblance to the amino-terminal lobe of a Cdk [16]. The physiological significance of this is currently unknown, but it is surely beyond coincidence. The key region involved in determining how the Cks folds up is a β hinge, comprising the residues HxPEPH (His–any–Pro–Glu–Pro–His) that are conserved throughout the Cks family. When this region folds back on itself the molecule forms a monomer, whereas with an extended β hinge the proteins dimerize (Fig. 2b). Cks proteins dimerize by exchanging β strands, an unusual type of protein–protein interaction that has so far been seen in only two other protein structures: seminal RNase and diphtheria toxin [17,18].

Three strand-exchanged dimers can in turn oligmerize to form a β barrel [2]. Although it is still unclear whether this change in conformation is ever seen *in vivo*, it would have dramatic consequences for the interaction between a Cks molecule and a Cdk. This can be inferred from the crystal structure of the binary Cdk2–CksHs1 complex, recently solved by Bourne *et al.* [5] (Fig. 2c). In this structure, CksHs1 is in its monomeric form, with the β hinge folded back on itself and forming important contacts with the Cdk. Furthermore, molecular modelling predicts that the Cks protein would be unable to bind to a Cdk when the β hinge is extended [5]. This is because the His and Glu residues of the Cks hinge region would be unable to contact the Cdk, and other key hydrophobic residues would be sequestered in an internal cavity of the dimer [3], or an internal channel of the hexamer [2]. In addition, the second subunit of the strand-exchanged dimer is predicted to be sterically hindered by the Cdk amino-terminal lobe [5]. Thus it is a formal possibility that in the cell there is an equilibrium between Cks monomers and dimers, in which only the monomers are able to bind to a Cdk. A change in this equilibrium would be predicted to have an effect on the cyclin–Cdk complexes, but the conditions that would alter the balance between monomers and dimers *in vivo* are not clear; *in vitro*, crystallization as dimers is favoured by the presence of Zn^{2+} [3,19].

Should the two forms of the Cks protein be able to interchange *in vivo*, this might allow for the intriguing possibility of a cell-cycle regulated conformational change from the monomer to the extended form. If this occurred at the end of mitosis, for example, it might force the Cks and Cdk to dissociate. In this regard it is interesting to note that a prolyl isomerase, which could play a role in altering the conformation of the β hinge, is essential for exit from mitosis in yeast, and is able to associate with another mitotic kinase, NimA, in a yeast two-hybrid genetic interaction screen [20].

Figure 2



The crystal structures of CksHs1 as (a) a monomer; (b) a strand-exchanged dimer; and (c) in a complex with Cdk2. CksHs1 is shown in yellow in all panels, and also in pink in (b) as the second component of the strand-exchanged dimer. The crucial residues that make up the β hinge (His 60 and Glu 63) are shown in their extended conformations in (b). In (c) the amino-terminal lobe of Cdk2 is shown in purple, and the carboxy-terminal lobe is shown mostly in blue; the α5 helix and L14 loop of Cdk2 that interacts with CksHs1 are shown in green. The T loop of Cdk2 is shown in light mauve/grey with the phosphorylated Thr 160 residue in orange; ATP is also shown binding between the two lobes. (Adapted from [5].)

Aside from raising the possibility of regulation by oligomerization, the crystal structures of the monomeric Cks and the Cdk–Cks complex do suggest clues to Cks function. The first feature to note is that the Cks subunit binds exclusively to a continuous sequence of residues that form α helix 5 and loop L14 at the carboxy-terminal lobe of the Cdk [5], far away from the cyclin-interacting region [21]. This means that a trimeric complex of cyclin–Cdk–Cks is perfectly feasible, in the case of either the G1 or the G2 cyclins in yeast — although it generates an as-yet unresolved question of why alanine-scanning mutagenesis should have implicated residues in the amino-terminal lobe of the Cdk as important for Cks binding [22].

In the budding yeast temperature-sensitive mutant *Cdc28-1N* [23], the L14 loop has a non-conservative point mutation in a conserved residue, Pro 250→Leu, which markedly reduces binding to Cks1p *in vitro* [5]. *Cdc28-1N* mutant cells arrest primarily in G2 phase, rather than in G1 phase like most other Cdc28 mutants, indicating that the interaction with Cks1 is especially important for the entry into mitosis in budding yeast. However, two mutations in fission yeast Cdc2 that map to this region give apparently contradictory phenotypes. The DL50 Cdc2 mutant [24] has a deletion of three amino acids (Leu–Gln–Asp, residues 240–242) and its phenotype is a lethal, premature entry into mitosis. In the crystal structure of Cdk2–CksHs1, the analogous residue to Cdc2 Asp 242 (Cdk2 Asp 235) makes a strong hydrogen bond *via* solvent to Glu 61, part of the hinge region of CksHs1, so by analogy the DL50 mutation would be predicted to have a deleterious effect on the Cdc2–Suc1 interaction. In contrast to the premature mitotic entry phenotype of DL50, a cold-sensitive mutant of Cdc2, in which Asp 242 is altered to Asn, causes a block in G2 phase and rescues the premature mitosis associated with overexpressing the Cdc25 phosphatase (which dephosphorylates and activates Cdc2) in cells lacking the Wee1 kinase [25]. Together with the genetic and biochemical results, therefore, the crystal structures support the idea that the interaction between the Cks proteins and Cdc2 (and perhaps the resulting effect on Cdc2 tyrosine phosphorylation) is crucial to passage through the G2–M-phase transition (see Fig. 1b).

The residues of loop L14 are evolutionarily conserved in the mitotic Cdks and in a subset of human Cdks: Cdk1, Cdk2 and Cdk3, but not Cdk4, Cdk5 and Cdk6. Thus, in animal cells, Cks proteins can only associate with a subset of the G1-phase kinases — cyclin A–Cdk2 and cyclin E–Cdk2 — explaining why affinity chromatography using Suc1 does not purify D-type cyclin–Cdk4/6 complexes. The L14 loop also has weak homology to a region in the MAP kinase family, and indeed MAP kinase activity has been found to bind to Suc1 affinity columns [26]. It is not clear, however, whether the cyclin–Cdk complexes that are purified on a Suc1

column bind because they initially lack a Cks subunit, or whether their original Cks subunit exchanges with the Suc1 on the column (the Cdk2–CksHs1 complex has a dissociation constant of 7.7×10^{-8} M), or whether more than one Cks subunit can bind each cyclin–Cdk complex.

A model of the predicted cyclin–Cdk–Cks complex reveals that the Cks would extend the site of interaction for a substrate of the kinase [5]. The ATP-binding site of the Cdk would be on the same side of the complex as a conserved, positively charged pocket of the Cks subunit that has been shown to bind the phosphate analogue, vanadate, in the crystal structure of CksHs1 [16]. This raises the possibility that the role of the Cks proteins is to target the cyclin–Cdk complex to phosphoproteins. Given that different proteins are phosphorylated at different times in the cell cycle, this might account for the apparently contradictory nature of a Cks deficiency in frog extracts and budding yeast compared with fission yeast, as suggested by Patra and Dunphy [14]. Thus, one phosphoprotein target in G2 phase could be the Cdc25 phosphatase that activates the cyclin B–Cdc2 kinase and triggers entry into mitosis. In contrast, the targets in mitosis might be phosphorylated components of the cyclin proteolysis machinery that inactivate the cyclin B–Cdc2 kinase and allow exit from mitosis. This still doesn't explain why adding excess Cks blocks Cdk dephosphorylation, however. Speculative explanations with no supporting data include: that more than one Cks monomer is able to bind a cyclin–Cdk complex but that this blocks access to phosphotyrosine 15 by the Cdc25 phosphatase; that excess Cks could drive the Cks equilibrium towards the strand-exchanged dimers that are unable to bind the cyclin–Cdk complexes; or that excess Cks protein could bind and sequester Cdc25.

The cyclin–Cdk–Cks model also has implications for the phosphorylation of the T-loop threonine residue that is required for full activation of the cyclin–Cdk complex. Access to the T-loop threonine appears to be restricted in the cyclin–Cdk–Cks complex, so there may be an order of assembly and disassembly to forming an active cyclin–Cdk complex. It may be that the T-loop threonine must be phosphorylated (by the specific cyclin-activating kinase, CAK) before the Cks is recruited, and that the T-loop threonine can only be dephosphorylated (by the specific phosphatase, KAP) after both the cyclin subunit has been destroyed and the Cks subunit has dissociated.

Thus there is still considerable confusion over the function of the Cks proteins, perhaps because of the apparent differences between organisms in the regulation of Cdk activity through tyrosine phosphorylation. The Cks and Cdk–Cks structures have gone some way towards suggesting what the Cks proteins could do — supporting the idea that they modulate interactions between Cdks and their regulators — but what they actually do still remains just out of reach.

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