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Review

The cdk-activating kinase (CAK): from yeast to mammals

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Abstract. Cell cycle progression is regulated by cyclin-dependent kinases (cdks). The activity of cdks is tightly controlled by several mechanisms, including binding of subunits to cdks (cyclins and inhibitors), and phosphorylation events. This review focuses on the activating phosphorylation of cdks by an enzyme termed cdk-activating kinase (CAK). Two classes of CAKs have been identified: monomeric Cak1p from budding yeast and the p40^{MO15}(cdk7)/cyclin H/MAT1 complex from vertebrates. Cak1p is the physiological CAK in budding yeast and localizes to the cytoplasm. p40^{MO15}(cdk7)/cyclin H/MAT1 localizes to the nucleus, is a subunit of the general transcription factor IIH and activates cdks as well as phosphorylates several components of the transcriptional machinery. Functions, substrate specificities, regulation, localization, effects on cdk structure and involvement in transcription are compared for Cak1p and p40^{MO15}(cdk7).

Key words. Cell cycle; cyclin-dependent kinase; cdk; cdk-activating kinase; phosphorylation; CAK; Cak1p; p40^{MO15}/(cdk7).

Introduction

Cell division is regulated by the sequential activation of cyclin-dependent kinases (cdks). Although the downstream targets of cdks are not well known [1, 2], phosphorylation of protein substrates by cdks is assumed to regulate most major events during the cell cycle. Upstream of the cdks, a network of regulatory mechanisms ensures proper timing of cdk activity. These mechanisms include protein-protein interactions (with cyclins, inhibitors and assembly factors), subcellular localization, transcriptional control, selective proteolysis and multiple phosphorylations (for reviews see [2–6]).

Binding of cyclins to cdks is essential for cdk kinase activity. Cyclins are unstable proteins that are synthesized and degraded periodically during the cell cycle [2, 7, 8]. Two mechanisms ensure proper timing of cyclin expression: transcriptional control regulates cyclin synthesis [9], and ubiquitin-mediated degradation of cyclins inactivates cdks irreversibly [4]. Cyclins are recognized by ubiquitin-protein ligases [APC (anaphase promoting complex) or SCF (Skp1p–Cdc53p/cullin–F-box protein)], poly-ubiquitinated and degraded by the 26S proteasome (for reviews see [10, 11]). In addition to cdk activation, cyclins are also likely to be involved in the substrate specificity of cdks [12, 13].

Cdk activity can be inhibited by tight binding protein subunits, termed cdk inhibitors (CKI or CDI, for review see [5]). In higher eukaryotes, two classes of CKIs have been identified: the CIP/KIP family consisting of p21$^{CIP1/WAF1}$ [14–16], p27^{KIP1} [17–19] and p57^{KIP2} [20, 21], and the INK family, including p15^{INK4b} [22], p16^{INK4a} [23], p18^{INK4c} [24, 25] and p19^{INK4d} [25, 26]. Members of the CIP/KIP family can associate with a

wide variety of cdks, but inhibit cdk2 better than cdk4 or cdc2 [19, 27]. The crystal structure of the $p27^{KIP1}$/cdk2/cyclin A complex confirmed that a single p27 molecule binds to both subunits [28], suggesting that one CKI molecule is sufficient to inhibit one cdk/cyclin complex. The N-terminus of p27 lies in the catalytic cleft of the cdk/cyclin complex, precluding the cdk from binding adenosine triphosphate (ATP) and substrates. The INK4 inhibitors are specific for cdk4 and cdk6 and bind to the cdk subunit [22–26, 29, 30].

Cdks are phosphorylated on several sites: two inhibitory and one activating phosphorylation site have been identified. The inhibitory phosphorylation sites (Thr-14 and Tyr-15 in human cdc2) keep cdc2 inactive until dephosphorylation by the dual specificity phosphatase cdc25, which initiates entry into mitosis. Thr-14 and Tyr-15 are phosphorylated by the myt1 and wee1 kinases (for review see [6, 31]). The third phosphorylation site was first identified in *Schizosaccharomyces pombe* cdc2 (Thr-167) and proven to be essential for activity of cdc2 [32]. Equivalent sites in *Xenopus* cdc2 (Thr-161) and *Saccharoymces cerevisiae* Cdc28p (Thr-169) are phosphorylated in vivo, are essential for cdk activity and for viability in budding yeast. These sites are phosphorylated by an enzyme termed the cdk-activating kinase (CAK). CAK in budding yeast was identified to be the monomeric Cak1p and in many other species to be the heterodimeric or heterotrimeric complex $p40^{MO15}$/cyclin H/MAT1, respectively.

Phosphorylation of cdks by CAK as well as cyclin binding changes the three-dimensional structure of cdks (for details see 'Activation of cdks'). Cyclin binding to cdk2 moves the T-loop (containing the activating Thr-160) from the 'closed' conformation to the 'open' conformation where the activating Thr-160 becomes accessible for CAK phosphorylation [33]. Phosphorylation of Thr-160 by CAK moves the T-loop further, enabling the phosphate group to interact with several residues, which stabilizes the structure of the T-loop [34].

This review will focus on CAK function, structural implications of cdk phosphorylation by CAK and CAK involvement in regulation of the cell cycle.

The players

Cak1p: *Saccharomyces cerevisiae*

In budding yeast, the 43-kDa Cak1p protein encoded by the essential *CAK1* gene (also called *CIV1*) was identified. Cak1p was discovered by biochemical purification monitoring CAK activity [35, 36], analysis of a Cdc28p associated protein (Civ1p) [37] and three genetic screens. In the genetic screens, *CAK1* displays synthetic lethality with the *SIT4* phosphatase [38], suppresses the spore wall morphogenesis defect of a conditional *smk1* mutant [39], and *cak1* mutant cells arrest with similar morphology as *cdc4*, *cdc34* or *cdc53* mutants [40]. However, none of these genetic screens revealed Cak1p's function as CAK. *SIT4* encodes the catalytic subunit of a protein phosphatase required in late G1 for normal accumulation of *CLN1* and *CLN2* transcripts [41]. *SMK1* (sporulation MAP kinase 1) encodes a developmentally regulated MAP kinase involved in the spore wall morphogenesis pathway and is essential for completion of sporulation [39, 42]. *CDC4*, *CDC34* and *CDC53* are subunits of the SCF^{Cdc4} ubiquitin-protein ligase and regulate the degradation of several cell cycle proteins (for review see [10]). The genetic interaction of all these genes with *CAK1* is difficult to interpret, but it could be that all these genes affect Cdc28p activity. Maybe there is even a direct or indirect physical interaction between Cak1p and these gene products.

Cak1p is a distant member of the cdk superfamily and shares only 20–25% identity with its closest homolog $p40^{MO15}$. The primary structure of Cak1p not only lacks the GxGxxG motif (domain L2, involved in nucleotide binding) but contains nonconservative replacements at 4 of 15 highly conserved amino acid positions in protein kinases. Only two other kinases, mik1 [43] and Vps15p [44], which lack all three glycines of the GxGxxG motif have been identified to date. Furthermore, Cak1p has several amino acid inserts between subdomains (between $\alpha 2$ and $\alpha 3$; $\beta 7$ and $\beta 8$; αL12 and L12; $\alpha 4$ and $\alpha 5$; and in L14). In contrast to cdks, Cak1p's in vitro activity towards cdk2 is not dependent on transactivating factors or posttranslational modifications, as expression in *Escherichia coli* produces an active kinase [35].

Overexpression of *CAK1* leads to elevated CAK activity [35], whereas immunodepletion of Cak1p from yeast extracts eliminates CAK activity [35, 36]. Cak1p coimmunoprecipitates with Cdc28p and vice versa, confirming the physical interaction of these two proteins [37]. Conditional mutant cells of *cak1* arrest at the restrictive temperature with 65% of the cells in G2 (*cak1-22*, 35% in G1 [35]) or with 70% of the cells in G1 (*civ1-4*, 30% in G2 [37]). These mutants arrest with low CAK activity, low Cdc28p activity and unphosphorylated Thr-169 in Cdc28p [35, 37]. Furthermore, *CAK1* genetically interacts with the major B-type cyclin, *CLB2* [35] and a G1 cyclin, *CLN2* [38]. *cak1-22* cells are unable to grow in a *Δclb2* [35] or a *Δcln2* [38] background at the permissive temperature, indicating that Cak1p regulates Cdc28p activity in G1 and G2. The temperature sensitivity of *cak1-22* is partially suppressed by expression of *CLB2* from a constitutively active promoter [35], but

not by expression of *CLN2* [38]. At the semipermissive temperature, *cak1-22* cells are also more sensitive to α-factor compared with wild-type cells [38]. These observations suggest that Cak1p is the physiological CAK in budding yeast and that it is involved in regulation of the G2-M and G1-S transitions.

p40^{MO15}(cdk7): mammals

CAK in mammalian organisms consists of the catalytic subunit p40^{MO15} (also called cdk7), a regulatory subunit cyclin H and an assembly factor MAT1. Therefore, mammalian CAK is also a cyclin-dependent kinase.

p40^{MO15} was originally identified as a negative regulator of oocyte maturation in *Xenopus* [45] and was subsequently shown to be a subunit of CAK in *Xenopus* [46, 47], humans [48–52], mice [53, 54], starfish [55], *S. pombe* (Mcs6, also called Crk1 or Mop1; [56, 57]), *Drosophila* [58], as well as a number of other organisms. Immunoprecipitation of human p40^{MO15} with a monoclonal antibody revealed stoichiometric binding of 37-kDa and 32-kDa proteins [48], later identified as cyclin H and the assembly factor MAT1 (see below). Cyclin H is closely related to the *S. cerevisiae* Ccl1p and the *S. pombe* Mcs2 (see below) and was identified by purification [53] and by a two-hybrid screen using p40^{MO15} as bait [59]. Unlike mitotic cyclins, cyclin H is expressed constantly during the cell cycle [48]. The crystal structure of cyclin H revealed similarity to cyclin A in the core domains. However, the N- and C-terminal regions of cyclin H and A are completely different [60, 61]. The assembly factor MAT1 (ménage à trois; [62–65]) displays little similarity to other proteins, except containing a C3HC4 putative zinc binding (or RING finger) domain, and promotes cyclin H binding to p40^{MO15} [62–64] as well as activation of p40^{MO15} ([63]; see 'Regulation'). Deletion of the RING finger domain does not affect the assembly or activity of CAK [64].

CAK-related enzymes

In *S. cerevisiae*, the enzyme most closely related to p40^{MO15} is Kin28p [66]. It is an essential protein kinase approximately 47% identical to p40^{MO15}. Kin28p associates with the cyclin Ccl1p [67, 68] and the assembly factor Tfb3p (also termed Rig2p; [69, 70]). Ccl1p shares similarity with cyclin H, and the *S. pombe* cyclin Mcs2 (see below). Furthermore, Kin28p is a subunit of the general yeast transcription factor IIH ([68, 71]; see 'CAK and transcription') where it phosphorylates the C-terminal domain (CTD) of the large subunit of RNA polymerase II. All of these features suggested that Kin28p is a functional homolog of p40^{MO15}. Nevertheless, Kin28p does not display any CAK activity [68, 72] but regulates transcription [71, 72].

In *S. pombe*, Mcs2 and Mcs6 are essential genes that were isolated as mitotic catastrophe suppressors of a *cdc2/wee1* double mutant [73] and display CAK as well as CTD kinase activities in vitro [56, 57]. Mcs2 is a cyclin that associates with the protein kinase Mcs6 (also called Crk1 or Mop1) that is homologous to p40^{MO15} [56, 57]. In addition, the nonessential protein kinase Csk1 (cyclin suppressing kinase) was isolated as a high copy suppressor of an *mcs2* mutation [74]. Deletion of Csk1 leads to a two- to threefold reduction of the Mcs2/Mcs6-associated kinase activity [74], implicating Csk1 in the regulation of Mcs2/Mcs6. Indeed, recent results indicate that Csk1 is the CAK-activating kinase (CAKAK) in *S. pombe* ([75]; D. Hermand and T. Mäkelä, personal communication).

In *Arabidopsis thaliana*, a high copy suppressor of a yeast *cak1* mutation (*civ1-4*) was identified. This gene encodes a protein termed Cak1At and is related to p40^{MO15}. By contrast, Cak1At shows no significant sequence similarity to the yeast Cak1p [76]. In addition to the suppression of the *civ1-4* mutation in *S. cerevisiae* [76], Cak1At suppresses also an *S. pombe mcs6-13/cdc2-3w/cdc25-22* mutation [76]. This result is surprising since p40^{MO15} cannot suppress a *cak1* mutation in *S. cerevisiae* [35]. Unlike p40^{MO15} and like *S. cerevisiae* Cak1p, Cak1At displays CAK activity but no CTD kinase activity [76]. Therefore, it is unclear if Cak1At belongs to the Cak1p or p40^{MO15} class of CAKs.

The game

CAK and transcription

p40^{MO15}, cyclin H and MAT1 are subunits of the general transcription factor IIH [77–80], and CAK is thought to phosphorylate the CTD of the large subunit of RNA polymerase II (see also below 'Substrate specificity'). A complete description of TFIIH has been published [81, 82]. p40^{MO15}, cyclin H and MAT1 bind to the core TFIIH through association with ERCC2 (XPD) [83, 84], and can also exist as a free complex dissociated from the core TFIIH [82]. In contrast, the yeast MAT1 homolog Tfb3p is a subunit of core TFIIH and might be the docking factor for TFIIK, which includes Kin28p and Ccl1p [68, 69, 85]. Although the concept of a direct connection between transcription and the cell cycle is tempting, the general requirement for CAK function in transcription has not been established. In vitro, CAK activity is not required for basal and activated transcription from the adenovirus major late promoter [86], or during initiation of transcription from the dihydrofolate reductase (DHFR) promoter [87]. However, it is required for transcription (elongation and/or promoter clearance) from the DHFR promoter [87]. These in vitro results indicate that CAK function in

transcription may depend on the promoter used, and we can only speculate about the situation in vivo (for review see [81]).

However, more information is available for the yeast system where TFIIH contains at least two different CTD kinases, Kin28p and Srb10p [88]. Both kinases phosphorylate the CTD with identical specificity, though phosphorylation of the CTD by Kin28p promotes transcription, whereas phosphorylation by Srb10p inhibits transcription [89]. This apparent contradiction is due to temporal regulation: Kin28p phosphorylates the CTD subsequent to preinitiation complex (PIC) formation; however, Srb10p phosphorylates the CTD prior to PIC formation.

Although transcription of many genes is dependent on Kin28p [71, 72], there are genes (e.g. *CUP1* and *SSA4*) that can be transcribed when Kin28p is inactivated [90]. These findings are similar to those reported for $p40^{MO15}$ (see above).

Substrate specificity

Most CAKs were purified by monitoring the activation of cdk/cyclin complexes in vitro [35, 36, 46, 47, 53, 55, 91, 92]. Two classes of in vitro substrates for $p40^{MO15}$ have been described: cdks and components of the transcriptional machinery. The cdk substrates include cdc2 [46, 47, 55, 91], cdk2 [46, 47, 53, 55, 92–95], cdk3 [92], cdk4 [27, 54, 96] and cdk6 [95, 97, 98]. Components of the transcriptional machinery that are $p40^{MO15}$ substrates include the CTD of the large subunit of RNA polymerase II [56, 57, 64, 68, 77–80, 99, 100], TFIIE [99, 100], TFIIF [100], the TATA-binding protein (TBP; [77, 99]), the retinoic acid receptor (RARα; [101]), the octamer transcription factor Oct-1 [102] and p53 [103, 104]. Phosphorylation of cdk4 was observed in extracts and immunoprecipitates [27, 54, 96] but not with purified proteins [95]. Perhaps cofactors are needed for efficient cdk4 phosphorylation by $p40^{MO15}$. The sites of phosphorylation for the different substrates are listed in table 1. The phosphorylation site in Oct-1 has not been described [102], and for p53 two different sites of phosphorylation, one near the N-terminus [103] and one near the C-terminus [104] have been reported. The residues around the phosphorylation sites differ in each substrate (tab. 1). Thus it was suggested that the residues around the phosphorylation site are less important for CAK specificity compared with the three-dimensional structure around the phosphorylated threonine (T-loop). With the identification of non-cdk substrates, this interpretation must be questioned since most of these substrates are phosphorylated at a serine instead of a threonine residue and additionally are not part of a T-loop. The most likely explanation is that $p40^{MO15}$ has a relaxed substrate specificity and more substrates will be identified in the future. Recently, the first in vivo substrate for *Drosophila* $p40^{MO15}$ was reported in cdc2 (see 'The score/comparison'; [58]). The same study also indicated that cdk2 is not a in vivo substrate of $p40^{MO15}$, which contradicts in vitro results.

The only confirmed in vivo substrate for a CAK enzyme is yeast Cdc28p, phosphorylated by Cak1p [35, 37]. Inactivation of *CAK1* in vivo leads to cell cycle arrest, decreased Cdc28p activity and loss of Thr-169 phosphorylation in Cdc28p [35, 37]. These experiments demonstrate the necessity of Cdc28p phosphorylation by Cak1p in vivo. Other reported in vitro substrates of Cak1p are cdc2 [105], cdk2 [35–37] and cdk6 [95]. To date, Cak1p has been found to phosphorylate cdks only and does not phosphorylate the CTD [35, 95].

A *cdc28* mutant with the activating Thr-169 mutated to a nonphosphorytable residue is not active, because Cdc28p activity is dependent on phosphorylation by Cak1p [35, 37, 72]. Using molecular evolution, a *cdc28* allele with T169E and several other mutations was created that is independent of the essential *CAK1* gene [106]. A yeast strain depending solely on this *cdc28* allele grows in the absence of Cak1p but not as well as a wild-type strain. Thus it was concluded that Cak1p's essential function is Cdc28p phosphorylation but that there might be other nonessential functions executed by Cak1p [106]. One of these nonessential functions of Cak1p could be phosphorylation and activation of Kin28p. Such a connection was suggested by a genetic interaction observed between mutations in *CAK1* and *KIN28* ([71]; J. Kimmelman and M. Solomon, unpublished), although inactivating *cak1-22* does not affect general transcription [38]. Inactivation of Cak1p

Table 1. $p40^{MO15}$ substrates.

Substrate										Position
cdc2	I	R	V	Y	**T**	H	E	V	V	165
cdk2	V	R	T	Y	**T**	H	E	V	V	164
cdk4	Q	M	A	L	**T**	P	V	V	V	176
cdk6	Q	M	A	L	**T**	S	V	V	V	181
CTD	(Y	S	P	T	S	P	$S)_x$			
RARα	P	S	P	P	**S**	P	P	P	L	81
p53	N	N	V	L	**S**	P	L	P	S	37
p53	K	**S**	K	K	G	Q	**S**	T	**S**	378

Substrates of $p40^{MO15}$ where the phosphorylation site was determined are listed. In the CTD it is conceivable that both threonine and serine are phosphorylated [142]. In the C-terminal part of p53, it has not been reported if all three serines (371, 376 and 378) or only one serine is phosphorylated by $p40^{MO15}$ [104]. The position of the last amino acid shown is indicated in the right column. References and accession numbers for the various substrates are as follows: human cdc2: [143], Y00272; human cdk2: [144], X61622; human cdk4: [145], S67448; human cdk6: [146], X66365; CTD: [147]; RARα: [148], X06614; human p53: [149], X01405.

changes the phosphorylation state of Kin28p in vivo [107, 108], and Cak1p phosphorylates Kin28p in vitro [107]. However, as for p40^{MO15} activation, the yeast MAT1 homolog Tfb3p might activate Kin28p in the absence of phosphorylation by Cak1p.

Substrates for CAK-related enzymes include the CTD for Kin28p [68, 71, 72], Mcs6 for Csk1 [75] and cdk2 for Cak1At [76]. None of the CAK enzymes phosphorylate the cdk substrates histone H1 and retinoblastoma protein (Rb). Substrate specificity is determined not only by the sequence and structure of the substrate but also by factors that bind to the substrates. Binding of cyclins to cdks was shown to stimulate cdk phosphorylation by p40^{MO15} [47, 53, 55, 91, 95]. p40^{MO15} does not phosphorylate monomeric cdc2 [53, 55, 91] and phosphorylates monomeric cdk2 only poorly [47, 53, 55, 95]. The presence of cyclin in a 1:1 ratio with cdk2 stimulates phosphorylation of cdk2 more than sevenfold [95]. Cyclin D3/cdk6 was phosphorylated by p40^{MO15}, whereas cyclin D1/cdk6 and cyclin D2/cdk6 were not, indicating that even different members of a cyclin family might change the structure of the cdk and therefore influence the ability of CAK enzymes to phosphorylate cdks [95]. These results might indicate the existence of different CAK enzymes that activate various cyclin/cdk complexes.

In contrast to p40^{MO15}, phosphorylation of cdks by Cak1p is not affected by CKI binding [95]. Furthermore, monomeric Cdc28p and cdk2 are efficiently phosphorylated by Cak1p [35–37]. In fact, cdk2 phosphorylation by Cak1p is dramatically decreased in the presence of cyclin A [95]. At a 1:1 ratio of cdk2 to cyclin A, phosphorylation by Cak1p is decreased approximately 90%, whereas phosphorylation by p40^{MO15} is stimulated sevenfold [95]. A similar effect is also observed for cyclin D3 and cdk6 [95]. These results demonstrate that Cak1p and p40^{MO15} belong to different classes of CAKs with specific substrate recognition patterns. Furthermore, these results suggest two alternative pathways for cdk activation (fig. 1). In the p40^{MO15} pathway, monomeric cdks first bind a cyclin, and this complex is phosphorylated and activated by p40^{MO15}. By contrast, in the Cak1p pathway, monomeric cdks are phosphorylated by Cak1p and subsequently bind a cyclin accompanied by activation of the cdk/cyclin complex. Possibly, both pathways could exist in the cell, explaining the need for two different CAK enzymes in one cell.

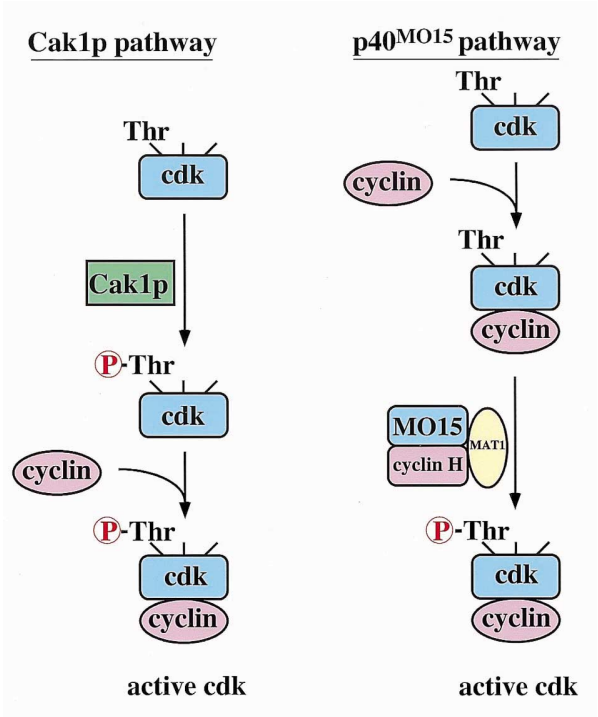

Figure 1. Alternative pathways for cdk activation. (Left column) Cak1p phosphorylates monomeric cdks which subsequently bind a cyclin to form the active cdk/cyclin complex. Cak1p activation of cdks takes place in the cytoplasm. (Right column) p40^{MO15} phosphorylates the pre-formed cdk/cyclin complex, resulting in a fully active cdk. This activation occurs in the nucleus. These models are based on in vitro data and will have to be confirmed in vivo. It is conceivable that both pathways exist in the same cell, explaining why two different CAK enzymes can be needed.

Regulation of CAK

Since CAK phosphorylation is essential for activation of cdks, an upstream cascade leading to CAK activation as in the MAP kinase pathway [109] might exist. Such a cascade has not yet been identified, and the regulation of CAK enzymes remains unclear. Protein levels and in vitro activity of p40^{MO15} are constant throughout the cell cycle [48, 49, 54, 64, 93, 110]. The same is true during early development in *Xenopus* [93, 94] and in quiescent cells [54, 94]. In vivo, p40^{MO15} is phosphorylated at Ser-164 and Thr-170 (residues in human p40^{MO15}, corresponding to Ser-170 and Thr-176 in *Xenopus* p40^{MO15}; [92, 93]). In the absence of MAT1, a T170A mutant of p40^{MO15} is inactive in vitro [53, 92, 93], whereas a S164A mutant retains activity [92, 93]. Regulation through phosphorylation at Ser-164 and Thr-170 is overlaid by the effect of MAT1 binding. MAT1 activates p40^{MO15}/cyclin H in the absence of Thr-170 phosphorylation [63]. Therefore, two alternative pathways to activate p40^{MO15} might exist in the cell. One pathway requires phosphorylation of p40^{MO15} on Thr-170 and the other pathway requires MAT1 binding only. Consistent with these in vitro results, a T170A mutant of p40^{MO15} can fully rescue a null allele in

Drosophila (S. Larochelle and B. Suter, personal communication), presumably through the function of a *Drosophila* homolog of MAT1. The physiological kinase(s) that phosphorylate $p40^{MO15}$ in vivo has not been identified, but cdk2 and cdc2 will phosphorylate $p40^{MO15}$ in vitro [63, 111]. In fission yeast, Csk1 activates Mcs6 (the *S. pombe* $p40^{MO15}$ homolog) in vitro and in vivo (D. Hermand and T. Mäkelä, personal communication). Csk1 is therefore the first identified CAK activating kinase (CAKAK). In the absence of Csk1, Mcs6 is possibly activated by a unidentified *S. pombe* MAT1 homolog.

MAT1 binding to $p40^{MO15}$/cyclin H stimulates CTD phosphorylation, whereas cdk2 phosphorylation decreases [99, 100]. In these experiments, monomeric GST-cdk2 was used instead of a cyclin/cdk complex, a much better substrate for $p40^{MO15}$ [53, 95]. CAK as part of TFIIH phosphorylates TFIIE and TFIIF in addition to the CTD, unlike recombinant $p40^{MO15}$/cyclin H/MAT1 [99, 100]. Therefore, MAT1 and other CAK interacting factors are likely to influence the substrate specificity in vivo.

The regulation of yeast Cak1p has not yet been studied in detail. In vitro, Cak1p activity and protein levels are constant throughout the cell cycle [36, 38] but decrease when cells enter stationary phase [112]. For at least minimal activity, Cak1p does not require posttranslational modifications or binding to regulators since expression of Cak1p in bacteria yields active enzyme [35]. This is corroborated by the observation that when gel filtration chromatography is performed on crude yeast extracts, Cak1p elutes as a monomer [112]. Cak1p in yeast extracts autophosphorylates to low levels [38, 40] when compared to phosphorylation of a substrate (cdk2). Mutation of potential phosphorylation sites in Cak1p has no effect on its activity in vivo or in vitro [112]. Furthermore, isoelectric focusing of Cak1p reveals no modified species, arguing that the majority of Cak1p molecules are not phosphorylated [112]. In addition, in vivo labeling of yeast cells with radiolabeled phosphate does not yield labeled Cak1p (unpublished result). These results do not exclude the possibility of short-lived phosphorylation or phosphorylation of a small fraction of Cak1p molecules.

In contrast to the vegetative cell cycle, *CAK1* messenger RNA (mRNA) [39] and protein levels [112] fluctuate dramatically during meiosis. This fluctuation predicts a short half-life of $\sim$1 h for Cak1p, which is different from its long half-life in the vegetative cycle [112]. When *CAK1* is expressed from the constitutively active *ENO1* promoter, Cak1p levels remained constant, and cells underwent sporulation in a normal fashion [112]. These results indicate that *CAK1* is regulated in meiosis by transcriptional control but that this regulation may not be essential for function. Nevertheless, in the absence of Cak1p, cells cannot complete sporulation [39].

Subcellular localization of CAK

Changes in subcellular localization can be an important feature in the regulation of cell cycle proteins like cyclins [113] and cdk inhibitors [114]. $p40^{MO15}$ is localized in the nucleus as shown by immunofluorescence microscopy [48, 64, 110, 115] and subcellular fractionation [48, 115]. The nuclear localization and subunit composition of $p40^{MO15}$/cyclin H/MAT1 is constant throughout the cell cycle [48, 115]. $p40^{MO15}$ colocalizes with TFIIH in coiled bodies (fig. 2A), dynamic subnuclear structures that may play a role in transport and/or maturation of small nuclear ribonucleoproteins (snRNPs) [116]. $p40^{MO15}$ also contains a strong nuclear localization signal (NLS; [117, 118]), and mutation of the NLS leads to cytoplasmic localization of inactive $p40^{MO15}$ [49, 92]. While the nuclear localization of CAK as part of TFIIH may be required for its function, it also agrees with the localization of cdk2, cdk4 and cdk6. However, the major mitotic cdk, cdc2, is predominately if not exclusively a cytoplasmic protein [119, 120] and only enters the nucleus together with cyclin B1 in prophase [119, 121, 122]. Therefore, it is unclear how $p40^{MO15}$ can activate cdc2, since cdc2 is already active when it enters the nucleus.

In contrast, budding yeast Cak1p is dispersed throughout the cell as was shown by immunofluorescence microscopy and confocal microscopy (fig. 2B). Subcellular fractionation suggested that most of the Cak1p protein is found in the cytoplasmic fraction [112]. Localization and fractionation of Cak1p are constant throughout the cell cycle. Localization of the major substrate of Cak1p, Cdc28p, was also reported to be predominantly cytoplasmic [123]. Therefore, activation of Cdc28p in yeast probably occurs in the cytoplasm. Nevertheless, Cdc28p (cdks in general) is found in excess over cyclins in the cell, most of the Cdc28p is monomeric and only a small portion of Cdc28p is cyclin bound [124]. In immunofluorescence experiments all Cdc28p molecules are detected, independent of whether they are monomeric or cyclin bound. However, only the cyclin-bound Cdc28p is active, and we do not know the function of monomeric Cdc28p. The cyclin-bound, active cdks may be found at a concentration too low to be detectable by immunofluorescence microscopy. Alternatively, Cdc28p might spend only a small fraction of its time within the nucleus and the majority of its time in the cytoplasm. Based on their involvement in DNA replication, Clb5p/Clb6p-Cdc28p complexes are likely to be found in the nucleus. Future studies should focus on the function and localization of active Cdc28p complexes or of inactive monomeric Cdc28p, respectively.

Activation of cdks

Structural analysis of cdks and other cell cycle proteins has advanced our understanding of cdk function enor-

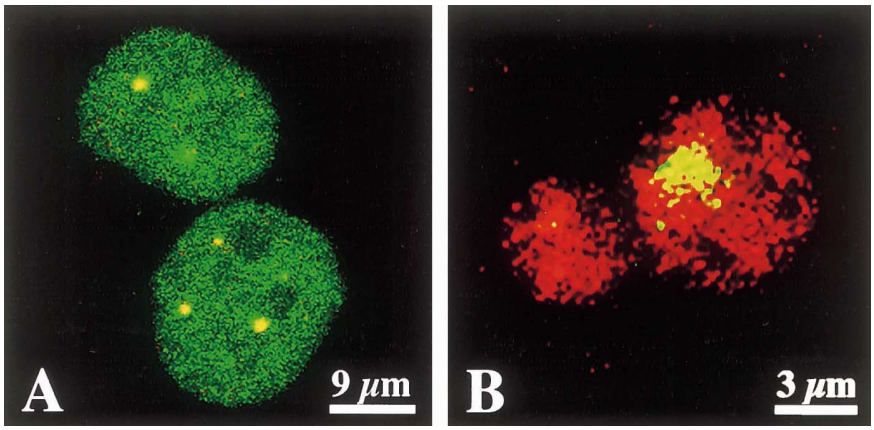

Figure 2. Localization of human $p40^{MO15}$ and yeast Cak1p. (*A*) Immunolabeling of HeLa cells using antibodies against $p40^{MO15}$ (green color) and against coilin (red color). Both proteins colocalize with coiled bodies (yellow color). HeLa cells were permeabilized with Triton X-100 prior to fixation in formaldehyde. Note that the nucleus is stained but not the cell body. This picture was kindly provided by P. Jordan and M. Carmo-Fonseca. (*B*) The subcellular localization of Cak1p was analyzed by confocal immunofluorescence microscopy including optical sectioning of single yeast cells. *S. cerevisiae* cells were stained both with the monoclonal antibody CAK1mAb1 (red) and polyclonal antibodies against the nuclear protein Mpp10p (green). Cak1p was localized throughout the cell (red color), whereas Mpp10p was found exclusively in the nucleus (yellow color). Note that the entire yeast cell (red) is stained, including the nucleus (yellow).

mously. The structure of cdk2 is divided in two lobes, a smaller N-terminal lobe consisting mainly of β-sheets and a larger α-helical C-terminal lobe [28, 33, 34, 125]. The two lobes are connected by a flexible hinge region, allowing movement between the lobes. Between the two lobes, there is the active site including the ATP binding site (P-loop or glycine-rich loop or glycine triad for the GxGxxG domain; [126, 127]) and the T-loop (also called activation loop or activation segment; [128]) containing the activating threonine. The T-loop is defined as the region spanning the conserved sequences DFG and APE, corresponding to residues 145–172 in cdk2. Close to the T-loop is the PSTAIRE helix which directly interacts with the cyclin subunit [33]. In the structure of monomeric cdk2, the T-loop faces the N-terminal lobe, and Thr-160 is buried [125]. This conformation is referred to as the 'closed' conformation. As mentioned above, cdk2 is activated by two events: binding to a cyclin and phosphorylation on Thr-160. These events affect the structure dramatically. Cyclin binding to cdk2 not only realigns the PSTAIRE helix but also moves the T-loop towards the C-terminal lobe [33]. When cyclin is bound, the T-loop is in what is described as the 'open' conformation, available for CAK phosphorylation. Phosphorylation of Thr-160 by CAK further moves the T-loop towards the C-terminal lobe and changes the conformation of the T-loop [34]. Binding of the p27 cdk inhibitor to the cdk2/cyclin A complex affects the structure of the T-loop in a minor way [28]. However, p27 significantly rearranges the N-terminal lobe of cdk2, and p27 inserts into the ATP binding site, preventing ATP binding. The crystal structure of p16/cdk6 and p19/cdk6 revealed that p16/p19 bind close to the ATP binding site of cdk6, on the opposite side where cyclin binds [29, 30]. p16 binding to cdk6 not only distorts the N-terminal lobe but also prevents ATP binding and causes a large movement of the T-loop [29]. This might explain why INK CKIs prevent phosphorylation of cdks by $p40^{MO15}$. The structure of monomeric cdk2 phosphorylated by Cak1p has been solved, and the T-loop structure appears with low electron density, suggesting that the T-loop might be very flexible in the absence of cyclin (J. Endicott, personal communication).

Structural evidence supports the model that cyclin binding is a prerequisite for CAK phosphorylation, since in the closed conformation Thr-160 is inaccessible. Furthermore, p27 binding to the cdk2/cyclin A complex appeared to sterically interfere with phosphorylation by CAK [28], explaining biochemical results (see above; [17, 95, 97, 129]). These conclusions were based largely on results obtained with $p40^{MO15}$. Recent data from experiments using yeast Cak1p draw a different picture. First, Cak1p efficiently phosphorylates monomeric cdks (in the closed conformation), and cyclin binding to cdks prevents phosphorylation by Cak1p [95]. Second, phosphorylation of cdks by Cak1p is not influenced by the presence of CKIs (both CIP/KIP or INK family CKIs) [95]. Likely explanations of these results could be that (i) Cak1p binding to cdks during phosphorylation

induces structural changes similar to those caused by cyclin binding (e.g. inducing the open conformation); (ii) Cak1p and cyclin binding sites on cdks are identical or overlapping, explaining the observed competition; and (iii) Cak1p approaches the Thr-160 on the T-loop from a different direction than $p40^{MO15}$ and is therefore unaffected by CKIs. These possibilities will need to be addressed in future studies.

Despite the large amount of data available, the requirement for Thr-160 phosphorylation in cdk2 by CAK has not yet been explained. Up until this point it has been assumed that Thr-160 phosphorylation is essential for the catalytic activity of cdks. Nevertheless, there have been indications that the catalytic activity of cdk2 might not (always) require Thr-160 phosphorylation: (i) Unphosphorylated cdk2/cyclin A complexes retain a low level of activity, in contrast to other cdk/cyclin complexes [34, 130, 131]. (ii) Phosphorylated and unphosphorylated cdk2/cyclin complexes display a similar amount of ATPase activity in the absence of substrate ([132]; R. Sheaff, D. Morgan and J. Roberts, personal communication). (iii) Phosphorylated and unphosphorylated cdk2, as well as the $cdk2^{T160A}$ mutant, can phosphorylate cyclin B [35] and p27 (R. Sheaff, D. Morgan and J. Roberts, personal communication). (iv) Both complexes phosphorylated a peptide substrate lacking a basic residue at the +3 position (relative to the activating threonine at position 0) to a similar degree [132], whereas unphosphorylated cdk2 phosphorylates a peptide substrate with a Lys or Arg residue at position +3 approximately 300 times less efficiently compared with phosphorylated cdk2.

These results together with the crystal structures suggest two possibilities for the function of the activating phosphorylation in cdks. One function involves the structure of the T-loop for catalytic activity, the other substrate recognition and/or binding. It was proposed that the phosphate group acts as an organizing center in the structure of the cdk2/cyclin A complex by interacting with several residues and therefore stabilizing the structure of the T-loop [34]. Other results suggest a role of Thr-160 phosphorylation in substrate recognition rather than in catalysis. Such a role might have been easily missed by using the model substrate histone H1 exclusively. Perhaps in vivo there exist a number of substrates that are recognized by unphosphorylated cdk2/cyclin complexes, especially substrates that bind tightly to cdks [13].

The score

Comparison

CAKs fall into two classes, the Cak1p-based class and the MO15-based class. The two classes of CAKs are highly divergent. Examination of their sequence, subunit composition, regulation, localization, substrate specificity and function in transcription indicates that the two enzymes have little in common. The only feature they share is that both Cak1p and $p40^{MO15}$ activate cdks by phosphorylating the activating threonine in vitro. By this criterion they have both been assigned as CAKs. So far, the physiological function of Cak1p has been clearly established. Cak1p is the (major) in vivo CAK of budding yeast Cdc28p (see above; [35, 37]). In contrast, $p40^{MO15}$ may have developed from a CAK into a multifunctional kinase involved in several cellular processes. The CAK function of $p40^{MO15}$ has been assumed for a long time, and only with the discovery of its involvement in transcription have doubts arisen as to its in vivo function. Much effort has been directed towards discussing the in vivo function of $p40^{MO15}$ [133–138] but there have been too few experiments that would allow conclusions. This complicated picture can probably only be resolved using systems that are genetically tractable, such as *S. pombe* or *Drosophila* (see below). In some organisms, two or more CAK enzymes might exist within one cell. This has been shown for *S. pombe* (Mcs6 and Csk1), where one enzyme functions upstream of the other [75]. Alternatively, additional independent CAK enzymes could differ in substrate specificity, localization or temporal expression. By contrast, budding yeast uses only one CAK enzyme, Cak1p, and the only $p40^{MO15}$ homolog, Kin28p, phosphorylates the CTD. More experimental studies are needed to compare the two classes of CAKs, especially in organisms where two different CAKs have been identified. Two recent reports support an in vivo function for $p40^{MO15}$. One used the *Xenopus* egg extract system and immunodepletion of $p40^{MO15}$ [139], whereas the other used the *Drosophila* system [58]. Larochelle et al. engineered a $p40^{MO15}$ knockout fly as well as a temperature-sensitive mutation in $p40^{MO15}$. $p40^{MO15}$ is an essential gene in *Drosophila*. The temperature-sensitive $p40^{MO15}$ flies display a phenotype very similar to cdc2 mutant flies [58]. Mutant flies show low cdc2/cyclin A and B activity at the restrictive temperature that can be restored by adding exogenous CAK, consistent with $p40^{MO15}$ being the in vivo cdc2-CAK in *Drosophila*. However, cdk2 activity was unaffected in these flies, suggesting the existence of an additional cdk2-activating kinase [58]. Clearly, more studies will be needed to investigate the identity of such a CAK specific for cdk2 in *Drosophila* and other organisms.

The upcoming game

Future

A reasonable amount of data has been collected about CAK enzymes, and yet more questions are raised than have been answered. It will be important to investigate

whether more than one CAK enzyme exists in cells. First indications suggests that this may be true not only for *S. pombe* but also for human cells (unpublished observation). If this is true, we will have to resolve the distinct functions of the various CAK enzymes. Do they differ in substrate specificity, localization or temporal expression? In budding yeast, Cak1p was suggested to be involved only in Cdc28p activation (essential function) and not in transcription. Nevertheless, since Cak1p phosphorylates Kin28p, it also indirectly regulates transcription. This connection between Cak1p and Kin28p as well as other functions of Cak1p need to be explored further.

Future studies using the *S. pombe* and the *Drosophila* systems may help clarify the in vivo functions of CAK. In *S. pombe* two different CAK enzymes have been identified (Csk1 and Mcs6), and Csk1 appears to be the CAKAK for Mcs6 (D. Hermand and T. Mäkelä, personal communication). However, Mcs6 has not been characterized in detail, and it is not certain that Mcs6 is the in vivo cdc2 CAK. The role of Mcs6 in transcription in *S. pombe* has not yet been assessed, except that it can phosphorylate a synthetic CTD peptide [56, 57]. In *Drosophila*, $p40^{MO15}$ was shown to activate cdc2 in vivo [58], and we hope that soon the as yet undiscovered cdk2 CAK will be found.

Furthermore, the question remains whether the activating phosphorylation needs to be removed from cdks at a given time. Such a phosphatase, KAP, has been identified in human cells and removes the phosphate from monomeric cdks but not from cyclin/cdk complexes [140]. The interplay of CAK and a phosphatase could introduce a temporal regulation of cdk activity. Nevertheless, there is no indication for a change in phosphorylation state in *S. cerevisiae* Cdc28p [141]. A definite answer will only come from the identification of such a phosphatase and the investigation of its function.

Note added in proof. A paper about *S. Pombe* CSK1 and Mcs6 has now been published: Hermand D., Pihlak A., Westerling T., Damagnez V., Vanderhaute J., Cottarel G. et al. (1998) Fission yeast Csk1 is a CAK-activating kinase (CAKAK). EMBO J **17:** 7230–7238

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