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The enzyme responsible for CAK activity in budding yeast is an essential cytoplasmic protein, Cak1p (22). Cak1p has no CTD kinase activity (23), indicating that the roles of Cdk7 in cell cycle control and transcription are separated in *Saccharomyces cerevisiae* into Cak1p and Kin28p, respectively. Although Cak1p bears sequence similarity to cdks, it is active as a monomer (requiring neither cyclin binding nor an assembly factor) and is not phosphorylated in *vivo* (24). Cak1p is an unconventional protein kinase in that its primary amino acid sequence lacks a number of conserved features. In particular, Cak1p lacks a canonical “glycine loop” in the nucleotide-binding fold and has substitutions in highly conserved core residues (23). Although it contains the “invariant lysine” essential for activity in all other protein kinases tested, this residue is completely dispensable for Cak1p activity both in *vivo* and in *vitro* (17, 25). Furthermore, Cak1p is insensitive to 5′-fluorosulfonylbenzoyladenosine (FSBA) (25), which covalently modifies the invariant lysine, leading to loss of activity in nearly all protein kinases, including Cdk2 (25) and Cdk7 (5). Both the glycine loop and the invariant lysine serve to position the ATP phosphates in protein kinases (26). Kinetic and mutagenic analysis demonstrated that Cak1p maintains high affinity for ATP despite its unusual sequence (25). In addition, the substrate specificity of Cak1p is different from that of Cdk7. Whereas Cdk7 prefers cyclin-cdk complexes, Cak1p prefers monomeric cdk substrates (27). This difference in substrate preference translates to different cdk activation pathways in *vivo*: Cdc28p, the yeast cell cycle cdk, is phosphorylated by Cak1p prior to cyclin binding (28) whereas vertebrate cdks bind cyclin prior to phosphorylation by Cdk7 (29).

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**Comparison of Cak1p-like Cyclin-dependent Kinase-activating Kinases**

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Cyclin-dependent kinases (cdks) coordinate progression through the eukaryotic cell cycle and require phosphorylation by a cdk-activating kinase (CAK) for full activity. In most eukaryotes Cdk7 is the catalytic subunit of a heterotrimeric CAK (Cdk7-cyclin H-Mat1) that is also involved in transcription as part of the transcription factor IHH complex. The *Saccharomyces cerevisiae* CAK, Cak1p, is a monomeric protein kinase with an atypical sequence and unusual biochemical properties compared with trimeric CAKs and other protein kinases. We sought to determine whether these properties were shared by a small group of monomeric CAKs that can function in place of CAK1 in *S. cerevisiae*. We found that *Schizosaccharomyces pombe* and *Arabidopsis thaliana* Cak1At, like Cak1p, all displayed a preference for cyclin-free cdk substrates, were insensitive to the protein kinase inhibitor 5′-fluorosulfonylbenzoyladenosine (FSBA), and were insensitive to mutation of a highly conserved lysine residue found in the nucleotide binding pocket of all protein kinases. The *S. pombe* and *C. albicans* kinases also resembled Cak1p in their kinetics of nucleotide and protein substrate utilization. Conservation of these unusual properties in fungi and plants points to shared evolutionary requirements not met by Cdk7 and raises the possibility of developing antifungal agents targeting CAKs.

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copy suppressor of an mcs2 mutant; Mcs2 is the cyclin partner of the S. pombe Cdk7 homolog Mca6 (30), which functions both as a CAK and as a subunit of TFIIF (31, 32). cskl does not rescue the lethal phenotype of a null allele of mcs2 and is a non-essential gene (30). Deletion of cskl results in a 3-fold reduction of Mcs2-associated kinase activity (30), is synthetically lethal with an mcs6 mutant (33), and causes a delay in reentry into the cell cycle (34). Furthermore, Csk1 is active as a monomeric protein kinase and phosphorylates Mca6 in vitro within the Mca6 T-loop, leading to its activation (34). These results point to a role for Csk1 as a CAK-activating kinase (34). cskl can rescue a cskl mutant in S. cerevisiae (35), and both Csk1 and Mcs6/Mcs2 may activate the S. pombe cdk Cdc2 in vitro, opening the possibility of redundant CAK functions (33).

The pathogenic yeast Candida albicans also has a protein that resembles Cak1p. This C. albicans gene product can complement a cakl mutation in budding yeast (37). A screen for Arabidopsis thaliana cDNAs able to complement a budding yeast cakl mutant yielded Cak1At, which was also able to complement a fission yeast cakl (mcs6) mutant (38). Cak1At resembles Cdk7 in sequence, phosphorylates Cdk2 within the T-loop in vitro, and exhibits no CTD kinase activity (38). The complementation of a cskl mutant suggested that Cak1At functioned as a monomer.

We have characterized the biochemical behaviors of the cakl-complementing protein kinases from S. pombe (Csk1), C. albicans (Cak1Ca), and A. thaliana (Cak1At). We were especially interested in establishing whether the properties that set Cak1p apart from the prevalent Cdk7-like CAKs were conserved in these proteins.

EXPERIMENTAL PROCEDURES

Yeast Culture and Manipulations—Yeast media and protocols were from Ausubel et al. (39). All yeast strains are based on W303-1A (MATa ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 sde1-d) (40) with the specified alterations.

Plasmids—The Cak1At gene was amplified by PCR from pYX112Cak1At (kind gift of M. Umeda) (38). A silent mutation was introduced to create an NcoI site at the start codon. The C. albicans Cak1 gene, henceforth referred to as Cak1Ca, was amplified by PCR from genomic DNA from strain CA14 kindly provided by M. Hoeter. Primers were designed based on the sequence derived by Faye et al. (37) except that the second amino acid was mutated from lysine to alanine in order to introduce an NcoI site for cloning purposes. The genes were subcloned into pVT4 (41), in frame with the GST enzyme and expressed in insect cells as a GST-CAK fusion protein produced at Stanford Genome Technology Center (based on strain SC5314). In-frame mutations (37) and differed slightly from a sequence in the genomic data base produced and maintained by the Stanford Genome Technology Center (based on strain SC5314). In addition, a phenylalanine to leucine mutation was inadvertently introduced at the second to last residue in the GSTCsk1 protein used in our in vitro studies. Because the protein was active in vitro and able to complement csklΔ in S. cerevisiae (data not shown), the change was deemed of little significance.

Protein Expression and Purification—1.5 × 10^9 Sf9 cells (Invitrogen) were infected with the appropriate baculovirus at a multiplicity of infection of ~5. 48 h postinfection the cells were harvested by centrifugation, and GST-tagged proteins were purified using glutathione-agarose (Sigma). Briefly, cells were resuspended in 5 volumes of lysis buffer (10 mM Hepes, pH 7.4, 10 mM NaCl, 0.5 mM EDTA, 0.1% Nonidet P-40, 10 mM DTT, and protease inhibitors (Sigma), with rotation for 45 min at 4 °C. The beads were washed with PBS, and protein was eluted in elution buffer (10 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0). The elution product was supplemented with ovalbumin to 0.5 mg/ml, glycerol to 10%, and protease inhibitors (see above) and stored at ~80 °C.

Biochemical Assays—The protein kinase activities of the purified GST-CAKs were assayed using human CDK2 produced in insect cells as a substrate (kind gift of A. Russo) (43). Each GST-CAK was incubated with 88 ng of CDK2 in the presence of 10 μM ATP and 5 μCi/μl [γ-32P]ATP in CASK buffer (50 mM Tris, pH 7.5, 15 mM MgCl2, 1 mg/ml ovalbumin, 0.5% Tween 20, and protease inhibitors as above) in a total volume of 20 μl. Reactions were incubated at room temperature for 15 min, stopped with the addition of 5× sample buffer, and analyzed by SDS-PAGE followed by autoradiography.

The effect of cyclin B on protein kinase activity was determined by carrying out kinase reactions as above except that CDK2 was preincubated with a 5-fold molar excess of a human cyclin A fragment (cyculin A173-412) (44). The results were quantitated using a Fuji Phosphorimage.

The K_m,CDK2 of each protein was determined as follows: 5 μl of an enzyme and protein substrate mixture (appropriate amount of GST-CAK and amount of CDK2 equal to 5 times the K_m,CDK2, except for Cak1At, in which case 47 μM CDK2 was used) was mixed with 5 μl of nucleotide (ATP varying in concentration from 180 to 0.08 μM at 0.06 μCi/μl in CASK buffer containing 150 mM NaCl). Reactions were carried out and analyzed as for K_m,CDK2 determination.

FSBA assays were carried out by incubating each GST-CAK with 1 μM FSBA or CDK2 with 20 μM FSBA in FSBA buffer (50 mM K+–Hepes, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mg/ml ovalbumin, and protease inhibitors). As a control parallel reactions were performed in which the enzyme and FSBA mixture contained 20 mM DTT to preactivate the FSBA. 5-μl aliquots were removed prior to and at various time points subsequent to FSBA addition. These were mixed with 5 μl of Cak buffer containing 20 mM DTT. To each reaction was added a 5-μl substrate mixture to assay for activity. For GST-CAKs this mixture contained 88 ng of CDK2, 10 μM ATP, and 0.5 μCi/μl [γ-32P]ATP in CASK buffer. In the case of Cak1At the substrate mixture contained 267 μM histone H1, 300 μM ATP, and 1 μCi/μl [γ-32P]ATP. Reactions were incubated at room temperature for 10 min, stopped with the addition of 5× sample buffer, and analyzed by SDS-PAGE and phosphorimaging.

RESULTS

To establish whether the unusual properties of Cak1p are conserved in CAKs from other species, we examined a group of protein kinases that can complement calΔ in S. cerevisiae (Fig. 1). The coding regions for S. pombe cskl, C. albicans Cak1, and A. thaliana Cak1At were subcloned into yeast expression vectors and also transposed onto bacmids from which recombinant baculoviruses were generated and used for protein expression in insect cells.

The ability of each of these genes to complement caklΔ suggested that the encoded proteins might function in the absence of regulatory subunits. To test this possibility, N-terminally GST-tagged fusions of the three protein kinases as
well as budding yeast Cak1p were expressed in Sf9 cells and purified using glutathione-agarose. Purification products were tested for their ability to phosphorylate CDK2 in vitro. All four proteins were active whereas no kinase activity was observed in a mock product from control uninfected insect cells (Fig. 2). We titrated the amount of each enzyme used in our assays so

**FIG. 1. CAK sequence alignment.** The protein sequence alignment of S. cerevisiae Cak1p (accession number 14318490), S. pombe Csk1 (accession number 544107), C. albicans Cak1 (accession number 3218550), A. thaliana Cak1At (accession number 125413) was manually adjusted after compilation using the Clustal algorithm in the MegAlign 4.00 sequence analysis software program (DNAStar Inc., Madison, WI). *Black shading* denotes sequence identity to Cak1p. Residues identical in other CAKs are shaded *gray*. The glycine loop motif is indicated by GxxG, and the invariant lysine residue is marked by an *asterisk*.

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that product yield versus enzyme concentration was in the linear range.

**Comparison of Kinetics**—Given the ability of these protein kinases to complement cak1Δ, we wanted to determine whether their kinetic parameters resembled those of Cak1p. We were especially interested in determining whether the yeast CAKs bind ATP efficiently in the absence of a well conserved protein kinase motif at the amino terminus of the nucleotide binding fold, the glycine loop (Fig. 1). This motif, usually GXGXXG, is typically required for effective anchoring of the ATP phosphates (reviewed in Ref. 26). Cak1p can function efficiently in the absence of this motif (25). The *S. pombe* and *C. albicans* proteins also lack a canonical glycine loop sequence (Fig. 1) (45). Using human CDK2 as a substrate we performed protein kinase assays at variable nucleotide and protein substrate concentrations to derive the \( K_m \) values for each kinase. Experiments were performed five times. Representative plots for each condition are shown in Figs. 3 (\( K_m(\text{CDK2}) \)) and 4 (\( K_m(\text{ATP}) \)), and the averages calculated in each case are summarized in Table I.

The values obtained for Cak1p agree with others previously derived in our laboratory: \( K_m(\text{ATP}) = 5.0 \mu M \) and \( K_m(\text{CDK2}) = 0.8 \mu M \) compared with 4.8 \( \mu M \) and 0.7 \( \mu M \), respectively (25). The *S. pombe* and *C. albicans* enzymes also exhibited low \( K_m(\text{ATP}) \) values of 8.5 \( \mu M \) and 1.8 \( \mu M \), respectively, demonstrating efficient nucleotide binding in the absence of a recognizable glycine loop. Because Cak1At had an immeasurably high \( K_m(\text{CDK2}) \) (greater than 15 \( \mu M \)) it was not possible to achieve saturating protein substrate concentrations in our experimental setup for the \( K_m(\text{ATP}) \) determination. The value of \( 60 \mu M \) is thus the apparent \( K_m \) for ATP of Cak1At at the subsaturating CDK2 concentration of 47 \( \mu M \). \( K_m(\text{CDK2}) \) values for Csk1 and Cak1Ca were 4.6 \( \mu M \) and 0.04 \( \mu M \), respectively.

**Monomeric CAKs Are Insensitive to FSBA**—All the enzymes under study here, including *S. cerevisiae* Cak1p, possess an invariant lysine residue, equivalent to Lys-72 in cAMP-dependent protein kinase (26) (see Fig. 1). This residue is conserved and required for activity in all tested protein kinases but is dispensable in Cak1p. To test the importance of this lysine in Csk1, Cak1Ca, and Cak1At, we treated these enzymes with FSBA, a nucleotide analog that covalently modifies the invariant lysine and inhibits the activity of all tested protein kinases other than Cak1p. After various durations of drug treatment, FSBA was inactivated with DTT, and the remaining protein kinase activities were determined. CDK2 is known to be FSBA-sensitive and was used as a positive control. The H1 kinase activity of CDK2 was completely inhibited after 30 min of treatment with FSBA (Fig. 5a, lanes 1–7, see also Ref. 25).
When FSBA was inactivated by preincubation with DTT, CDK2 retained activity even after treatment (Fig. 5a, lanes 8–14). The immediate reduction in activity in the presence of inactivated FSBA (Fig. 5a, lane 9) was due to the ability of FSBA to compete with ATP for the nucleotide binding pocket of CDK2 (25). This effect did not increase with incubation time.

As previously shown, budding yeast Cak1p is relatively insensitive to treatment with FSBA at a concentration 50 times higher (1 mM) than was sufficient for complete inhibition of CDK2 (20/262M) (compare Fig. 5, a and b). Some Cak1p activity remained at 120 min of incubation with FSBA, whereas CDK2 was completely inactivated by 30 min in the presence of much less FSBA. Like Cak1p, Csk1, Cak1Ca, and Cak1At were also insensitive to FSBA and retained ~50% activity through 2 h of drug treatment.

**Mutation of the Invariant Lysine**—The insensitivity to FSBA suggested that the position and role of the invariant lysine in the ATP-binding pocket of these CAKs was unusual. To determine whether this lysine is dispensable, as in Cak1p, we mutated the corresponding lysine in each protein (Fig. 1) to alanine and introduced the mutant gene into a yeast strain to evaluate its ability to complement cak1Δ. Briefly, a CAK1/cak1::HIS3 diploid strain was transformed with the lysine mutant genes (denoted by the superscript KA in Fig. 6) or their wild-type versions on plasmids, and the cells were induced to sporulate. Tetrad were dissected, and the resulting colonies containing the CAK1 deletion and the plasmid were identified. All the lysine to alanine mutants rescued cak1Δ lethality and grew at rates similar to cak1Δ strains rescued by the corresponding wild type gene under normal laboratory growth conditions (Fig. 6).

**Cyclin Inhibition of Monomeric CAKs**—The metazoan CAK...
Cdk7/cyclin H favors cyclin-bound cdk substrates, whereas the budding yeast Cak1p prefers to phosphorylate monomeric cdks (27). We investigated the effect of cyclin on the activities of Csk1, Cak1Ca, and Cak1At. Each enzyme was assayed for CAK activity in the absence of cyclin A (defined as 100% activity) or in the presence of a 5-fold molar excess of cyclin A173–432 to CDK2. As a control, we used denatured (boiled) cyclin A. Activities were expressed as a percentage of the activities obtained in the absence of cyclin. The averages of three (native cyclin) and two (boiled cyclin) independent experiments are plotted in Fig. 7. All four CAKs showed a strong preference for cyclin-free cdk (no cyclin or with addition of denatured cyclin). Cyclin-CDK complex formation reduced phosphorylation by ~90% for all four CAKs, similar to the inhibition previously observed for the phosphorylation of CDK2 by Cak1p (27).

**DISCUSSION**

Cyclin-dependent kinases are integral components of the cell cycle apparatus in eukaryotes and are subject to conserved regulatory mechanisms. The requirement for activating phosphorylation is a conserved element in the regulation of cdks. Cdk7, the catalytic subunit of the trimeric CAK, appears to have an ortholog in all species. Identification of Cak1p as the only CAK in the cell.

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Cak1p-like CAKs

lysine to alanine. That the KA mutants are functional in vivo indicates that they are not severely compromised in ATP utilization or CAK function. It is possible that another residue has assumed the role of the invariant lysine in nucleotide phosphate alignment or that these protein kinases fold into a structure wherein the ATP-binding pocket allows for productive orientation of the ATP molecule without the requirement of a single critical residue. These findings are particularly surprising in the case of Cak1At as its primary sequence carries the highest degree of identity with Cdk7.

The fact that Csk1, Cak1Ca, and Cak1At are active when expressed in insect cells suggests that all are active in the absence of binding partners. This result is surprising in light of the sequence similarity of Cak1At to Cdk7. Although it is possible that Cak1At activity might be increased by a cyclin partner, Cak1At is able to function satisfactorily without one. In inactive monomeric cdkks, the T-loop blocks the active site and inhibits kinase activity. Cyclin binding causes conformational changes, including repositioning of the T-loop, that activate its cdk partner (44). A large insertion in the Cak1At T-loop (compared with the T-loop region of Cdk7 and other cdkks (45), see Fig. 1) might function to maintain an active conformation, bypassing the requirement for cyclin binding. Another plant, Euphoria esula, possesses a Cdk7 homolog (accession number 6984233) that shares high sequence similarity with Cak1At and includes a long T-loop of approximately equal length. Within this insertion there is extensive variation between the two plant proteins with a region of increased identity proximal to the putative site of activating phosphorylation (Thr-290 in Cak1At, Xenopus Cdk7 and S. pombe Mcs6) can be active as monomers under special conditions (33, 47), and even Cdk2 can have low but measurable activity as a monomer (48). Cak1At may represent a more extreme version of this tendency.

Finally we found that Csk1, Cak1Ca, and Cak1At, like Cak1p but unlike Cdk7, all exhibit a preference for cyclin-free cdk substrates. For Cak1p, this substrate preference conforms with a cdk activation pathway in S. cerevisiae in which activating phosphorylation of Cdc28p precedes cyclin binding (28). In contrast, the preference of Cdk7 for cyclin-cdk complexes supports an activation pathway in which cyclin binding precedes activating phosphorylation. Applying the same logic to Csk1, Cak1Ca, and Cak1At, we might expect that activating phosphorylation precedes cyclin binding in S. pombe, C. albicans, and A. thaliana.

It is unclear what advantage one activation pathway may have over the other. It is possible that they are equivalent alternatives accomplishing the same effect, the activation of cyclin-dependent kinases. If this is the case, however, it becomes increasingly difficult to explain why both monomeric and trimeric CAKs have been conserved, sometimes in the same species. S. pombe possesses the Cak1p-like CAK Csk1 as well as a Cdk7-type CAK, Mcs6 (33, 34). Similarly there is evidence of Cdk7-like CAKs in A. thaliana (38, 49), and rice also appears to have a heterotrimeric Cdk7-like CAK (50). Retention of a monomeric CAK suggests that these organisms share a common requirement that is not satisfied by Cdk7. This may involve Cdk7 regulation, as would be the case if the Cak1p homolog acts as a CAK-activating kinase or it may be Cdk7-independent. In budding yeast there is evidence that Cak1p is involved in meiosis and spore formation (36, 51). Conservation of a Cak1p-like mechanism in other yeast (C. albicans and S. pombe) and plants (A. thaliana) may point to a common requirement for a Cak1p-like CAK in yeast sporulation and gamete formation in plants. It is perhaps significant that these organisms possess cell walls. Cak1p then may have evolved as the CAK able to act both in the regular cell cycle, as does Cdk7, as well as in pathways specific to these organisms. The three yeasts, but not the plant A. thaliana, undergo closed mitoses, so it is also conceivable that the non-Cdk7-like CAKs are important in cells in which nuclear and cytoplasmic contents do not mix during mitosis. It will be interesting to see if Cak1p-like CAKs are found in other yeast and plant species.

To date, no Cak1p ortholog has been identified in metazoar. In our studies, however, we have found that the monomeric kinase Cak1At resembles Cak1p in substrate specificity, insensitivity to FSBA, and lack of requirement for the invariant lysine. It remains possible, therefore, that a monomeric CAK exists in some metazoar, bearing sequence similarity to Cdk7 and biochemical similarity to Cak1p. If on the other hand the Cak1p-type CAKs are a family of CAKs unique to yeast and plants, then their distinct structure and atypical ATP-binding properties may render them selectively sensitive to a specially designed inhibitor. As such, Cak1p-like CAKs might provide a potentially useful antifungal drug target.

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