p42, a Novel Cyclin-dependent Kinase-activating Kinase in Mammalian Cells*

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The cyclin-dependent kinase (CDK)-activating kinase (CAK) phosphorylates a conserved threonine residue on CDKs and activates them. Two known classes of CAKs are represented by monomeric Cak1p in budding yeast Saccharomyces cerevisiae and by heterotrimeric CDK7-cyclin H-Mat1 in human and other metazoans. We report here the identification of p42, a novel CAK activity in human cells. p42 has sequence homology to both Cak1p and CDK7 groups of CAKs. p42 is essential for the phosphorylation of Thr-160 and activation of CDK2. A dominant-negative p42 mutant, T161A, and posttranscriptional gene silencing of p42 with RNAi-impaired Thr-160 phosphorylation and activity of CDK2. Purified p42 phosphorylated glutathione S-transferase-CDK2 at Thr-160 within the T-loop and activated its histone H1 kinase activity. Finally, p42 is indispensable for cell growth. Cells lacking p42 were incapable of growing and forming colonies whereas cells with a reduced level of p42 grew at significantly slower rates than control cells. Our findings suggest that p42 represents a novel CAK activity in mammalian cells.

Cell cycle transitions are controlled by a family of cyclin-dependent protein kinases (CDKs)† (1, 2). Activities of CDKs are tightly regulated by multiple mechanisms, including association with cyclins, negative regulatory subunits, and phosphorylation. Inhibitory phosphorylation of CDKs on N-terminal threonine and tyrosine residues (Thr-14 and Tyr-15 on CDK2) by the WEE1 family of protein kinases is counteracted by the action of CDC25 phosphatases, which are essential for dephosphorylating and activating CDKs. In the absence of inhibitory phosphorylation on Thr-14 and Tyr-15, the CDK subunit remains inactive. The interacting cyclin subunit and an activating phosphorylation on the conserved threonine residue within the T-loop region on CDK are required for the complete activation of CDKs. CDK-activating phosphorylation occurs on Thr-161 in human CDC2 and Thr-160 in human CDK2. Phosphorylation of Thr-160 by CDK-activating kinase (CAK) promotes structural changes in the T-loop and increases CDK2 kinase activity by several orders of magnitude (3).

With the clear importance of CAK phosphorylation in mind, much effort was devoted to the identification and characterization of CAK. A rather complicated picture emerged. Biochemical identification of CAK in Xenopus and human cell extracts resulted in the characterization of CAK as a tripartite complex among CDK7, cyclin H, and Mat1 (4–9). Additionally, CDK7-cyclin H complexes are a part of a TFIIH, a transcription factor required for the initiation of transcription and for DNA repair (10, 11). CDK7 is involved in phosphorylation of the C-terminal domain of the large subunit of RNA polymerase II required for the elongation step during transcription (12, 13).

In the budding yeast S. cerevisiae, a different kinase, Cak1p, is an essential CAK. Monomeric Cak1p does not require a cyclin partner for its function (14–16). C-terminal domain phosphorylation in the budding yeast is performed by another kinase, Kin28p, which has been shown not to be required for Cdc28 phosphorylation in vivo and cannot act as CAK in vitro (17). In fission yeast, CAK function is controlled by the CAK network that consists of an Mcs6 (CDK7 ortholog)-Mcs2 complex (18–20) and the second CAK, Csk1 (21–23). Csk1 does not require a cyclin partner for its function and has other biochemical characteristics similar to the budding yeast Cak1p.

The discovery of the budding yeast Cak1p prompted the re-evaluation of the role of CDK7 in metazoas. In a fruit fly Drosophila melanogaster strain expressing the temperature-sensitive Cdk7 mutant, phosphorylation of Thr-161 in Cdc2 was lost at the restrictive temperature, providing strong evidence that CDK7-cyclin H is essential at least for phosphorylation of CDC2 in a metazoan organism (24). Surprisingly, the temperature-sensitive mutation in CDK7 did not reveal the CDK7 requirement for CAK activity toward cyclin E-CDK2 in the fruit fly. Moreover, a dominant-negative CDK7 mutant did not affect mitotic divisions in Drosophila (25). Somewhat in contradiction to the above, in the nematode Caenorhabditis elegans, CDK7 is essential for mitotic and meiotic progression, and complete ablation of CDK7 by RNAi causes embryos to arrest at the one-cell stage (26). In human cells, a role for an alternative CAK (p45) in the activation of CDK2 has been suggested (27), but its nature remains elusive despite concerted efforts to purify this activity.

EXPERIMENTAL PROCEDURES

Antibodies—A 12-amino acid C-terminal p42 peptide fused to keyhole limpet hemocyanin was used to raise anti-p42 antibodies in rabbits. The resulting antiserum was affinity-purified with antigenic peptide immobilized on SulfoLink coupling agarose (Pierce). The purified anti-p42 antibodies were stored at 0.4 mg/ml and used at various dilutions throughout this study. Anti-FLAG (M2, Sigma), anti-hemagglutinin (HA) (16B12, Covance), anti-CDK2 T160 phospho-specific antibodies (Cell Signaling Technology, Inc.), and anti-glycerolaldehyde-3-phosphate dehydrogenase antibody (Chemicon) were used according to the manufacturers’ suggestions.

Recombinant Proteins—Recombinant His6-p42 was produced and purified using a Bac-to-Bac system (Invitrogen) following the manufacturer’s instructions. The wild type and mutant glutathione S-transferase (GST)-CDK2 constructs were gifts of Dr. R. Poon. GST-CDK2K33R is a kinase-dead mutant whereas GST-CDK2T160A has Thr-160 substi-

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‡ The abbreviations used are: CDK, cyclin-dependent kinase; CAK, CDK-activating kinase; Csk1, cyclin-dependent kinase-degrading activity; CDC25, cyclin-dependent kinase phosphatase; CAK, CDK-activating kinase; Cak1p, cyclin-dependent kinase-activating kinase; Cdc2, cyclin-dependent kinase-2; Csk1, cyclin-dependent kinase-degrading activity; CDC25, cyclin-dependent kinase phosphatase; HA, hemagglutinin; GST, glutathione S-transferase; WCE, whole cell extract; siRNA, small interfering RNA; IP, immunoprecipitation.
tuted by an unphosphorylatable alanine. These were purified as described elsewhere (4).

Cell Culture and Synchronization—All cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were regularly passaged to maintain exponential growth. A double thymidine block-release was used to synchronize HeLa cells. Briefly, cells were cultured in the presence of 2.5 mM thymidine for 17 h, released to media free of thymidine for 9 h, and cultured again in the presence of 2.5 mM thymidine for 15 h. The resulting G1/S boundary-arrested cells were then released to achieve synchronization. At 2-h intervals, cells were collected for Western blotting and flow cytometry cell cycle profile analysis.

Immunofluorescence—Cells grown on coverslips were fixed with 1% formaldehyde and permeabilized with 0.1% Nonidet P-40. Nonspecific binding sites were blocked with 10% normal goat serum. The slides were mounted with 4’,6-diamidino-2-phenylindole hydrochloride containing mounting medium (Vector Laboratories, Inc.).

Small Interfering RNA (siRNA) and Stable RNA Interference—siRNAs targeting the human p42 cDNA sequence GGCGGTTGGAGGACGGCTT, corresponding to positions from +113 to +121, were synthesized by Dharmacon Research. For annealing siRNAs, 20-μM H9262 siRNA duplex was transfected into cells using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s instructions. For each well of a 6-well plate, 2 μg of siRNA duplex was transfected.

A pSuper-based plasmid, pSuper-p42, was constructed that directs the synthesis of siRNA once transfected into cells (28). pSuper-p42 was designed to target the same mRNA sequence as p42-2 siRNA. pSuper-p42 was co-transfected with pBABEpuro (at a 1:10 ratio) into HeLa cells using LipofectAMINE plus (Invitrogen). Forty-eight hours posttransfection the cells were fed with a medium containing 1 μg/ml puromycin. The selection was kept until the resistant colonies reached the size of more than 100 cells (~10–14 days). Finally, the morphology of live colonies was captured using an Olympus IX70 inverted microscope with SPOT software (Diagnostic Instruments, Inc.), and the plates were fixed and stained with 1% crystal violet in 20% ethanol.

CDK2 Kinase Assay, CAK Assay, and CDK2 Activation Assay—To test CDK2 and CDK7 kinase activity, WCE were immunoprecipitated using anti-HA (for either HA-CDK2 or HA-CDK7 immunoprecipitation), anti-CDK2, or anti-CDK7 antibodies. Immunoprecipitates were washed four times with an IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 20 mM NaF, 1 mM NaVO4, 20 μM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, and 1 μM leupeptin), and the resultant pellet was washed twice more with kinase buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 20 mM EGTA, 20 mM β-glycerophosphate, 10 mM MgCl2, 5 mM MnCl2, and 5 mM DTT). CDK2 kinase assay was assayed in the presence of 2 μg of histone H1, 100 μM ATP, and 10 μCi of [γ-32P]ATP in kinase buffer at 30 °C for 20 min. CDK7 activity assays were carried out under similar conditions using GST-CDK2 as a substrate. The reactions were stopped with an equal volume of 2× SDS loading buffer. Samples were boiled for 2 min and loaded onto 10% SDS-PAGE. The signal was processed with a Storm 860 PhosphorImager system (Amersham Biosciences).

The CAK activity of human p42 from two sources was tested. One microgram of anti-p42 in the presence or absence of the competitive peptide was used to immunoprecipitate HeLa WCE with endogenous or overexpressed p42. The kinase activity associated with p42 immunoprecipitates was assayed using 500 ng of GST-CDK2 as the substrate in the presence of [γ-32P]ATP. To test the CAK activity of purified recombinant p42, we used 200 ng of purified His6-p42 as the kinase source with two mutants of GST-CDK2, namely GST-CDK2K33R and GST-
CDK2T160A as the substrates. The assay was performed as described above.

For CDK activation assays, 500 ng of bacterially produced GST-CDK2 protein was bound to 20 μl of glutathione Sepharose beads (Amersham Biosciences) in a bead-binding buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 0.2% Nonidet P-40) at room temperature for 30 min. After two washes with the same buffer, the beads were incubated with 200 ng of purified His8-p42 protein or with an equal volume of mock-purified a9 cell WCE in the presence of 1 mM ATP in a kinase buffer at 30 °C for 30 min. The reaction was terminated by washing away His8-p42 protein with bead-binding buffer. Histone H1 kinase activity associated with the beads was then assayed as previously described.

RESULTS AND DISCUSSION

Early attempts to identify yeast Cak1p ortholog(s) in mammalian cells using bioinformatics and/or biochemical approaches were unsuccessful. We argued that it might be possible to identify the potential budding yeast Cak1p ortholog in human cells by using the BLAST search of lower eukaryotic genomes and then comparing the resultant molecules to human and mouse genomes. For this purpose we used the Cak1p amino acid sequence and searched the D. melanogaster protein data base using BLAST. As a result, we identified a previously uncharacterized kinase (CG6800, GenBank™ AAF55917) among the top five BLAST “hits.” The same putative kinase was a top BLAST hit when D. melanogaster genomic data base was searched using BLAST and the Schizosaccharomyces pombe Csk1 protein sequence. Because both Cak1p and Csk1 are bona fide CAKs in budding and fission yeasts, we decided to investigate this homology further. A comparison of CG6800 back to the Drosophila genomic data base established that the closest homolog of CG6800 in Drosophila is CDK7, further suggesting a potential connection between an additional putative metazoan CAK activity and CG6800. To investigate this further we again used BLAST to compare CG6800 conceptually translated protein and the human genomic data base. A single best match was with the cell cycle-related kinase (GenBank™ AAN28884), also called CDK-related protein kinase FNQLARE (GenBank™ AAF89089) followed by a second best match to the human CDK7. Because this putative kinase showed clear homology to both CDK7 and to the Cak1p/Csk1 group of kinases in evolutionary diverged species (Fig. 1), we decided to investigate it further. A full-length human cDNA corresponding to cell cycle-related kinase/PNQLARE was cloned by PCR from a HeLa cDNA library and subcloned into pcDNA3.1 mammalian expression vector. In vitro translation of this cDNA resulted in a protein with a relative mobility on a 10% polyacrylamide gel, providing further evidence for the specificity of the antibody (Fig. 2 right panel). The pres-
**Fig. 3.** p42 is essential for CDK2 activity and phosphorylates CDK2. **a**, dominant-negative p42 impairs CDK2 activity. Upper panel, plasmids encoding FLAG epitope-tagged wild type or mutant p42 were co-transfected into U2OS cells with HA epitope-tagged CDK2. Forty-eight hours posttransfection cells were lysed and subjected to immunoblot analysis with anti-FLAG, anti-HA, and anti-CDK2 phospho-specific antibodies. CDK2-HA was first immunoprecipitated with anti-HA antibody and IP-associated histone H1 kinase activity was assayed in the

**b**

| siRNA: | EGFP | p42-2 |
|-------|------|-------|
|        | p42  | k2    |
|        | k2/H1| k2 phospho |
|        | k7   | k7/GST-CDK2 |

**c**

| Percentage | EGFP siRNA | p42 siRNA |
|------------|------------|------------|
| G1         | 80         | 70         |
| S          | 20         | 30         |
| G2/M       | 5          | 20         |

**d**

|          | mock | His6-p42 | H1 |
|----------|------|----------|----|
|          | -    | -        | -  |
| peptide | ed   | +        | -  |
|          | +    | -        | +  |

|          | mock | His6-p42 | GST-CDK2 |
|----------|------|----------|----------|
|          | -    | -        | -        |
| peptide | ed   | +        | -        |
|          | +    | -        | +        |

[Caption: p42, a Novel CAK Activity]
A characteristic feature of budding yeast Cak1p is that it exists as a monomeric protein (14, 15). To determine whether p42 exists as a monomer, HeLa cell extracts were separated by gel filtration, and p42 was detected by immunoblotting. As a result, p42 was detected in fractions corresponding to a molecular mass of ~40 kDa and a leading peak (on the edge of void volume, ~60 kDa) (Fig. 2). Leading peaks are sometimes artifacts of protein aggregation, so p42 is most likely a largely monomeric in cells, mimicking budding yeast Cak1p.

If p42 functions in vivo as a CAK, its dominant-negative version should interfere and therefore cause lack of phosphorylation of the conserved T-loop threonine in CDKs (e). To test this hypothesis, two point mutations were introduced into p42 in an attempt to make dominant-negative mutants. Plasmids encoding FLAG epitope-tagged wild type or mutant p42 together with plasmids encoding influenza HA epitope-tagged CDK2 were co-transfected into U2OS cells. Interestingly, K33T/K34T, T161A, and wild type p42 were not expressed at similar levels with Thr-161 being expressed at a significantly lower level than the other two. Expressed CDK2 kinases were immunoprecipitated from WCE using anti-HA antibody and assayed against histone H1. We found that ectopically expressed CDK2 activity was significantly compromised when T161A was co-expressed (Fig. 3a, top panel). This result is consistent with a previous report that the change of the T-loop threonine in CDK7 to alanine had a dominant-negative effect (25). With an anti-CDK2 Thr-160 phospho-specific antibody, we were able to show that the compromised CDK2 activity was accompanied by a decrease in CDK2 Thr-160 phosphorylation (Fig. 3a, top panel). The reduction of CDK2 activity was not the result of altered CDK2 protein abundance because as immunoblotting demonstrated the expression levels were similar. Transient co-expression of wild type p42 had no effect on ectopically expressed CDK2 activity, suggesting that if p42 activates CDK2 the endogenous protein is sufficient. K33T/K34T failed to show any dominant-negative effect. In a parallel experiment, HA-tagged CDK7 together with FLAG-tagged p42 were co-transfected into U2OS cells. Neither wild type nor mutant p42 affected the abundance or activity of the co-expressed CDK7, demonstrating that p42 has no impact on CDK7 activity in vivo (Fig. 3a, bottom panel).

To analyze p42 function further, we used siRNA to achieve posttranscriptional gene silencing. A 21-bp double-stranded siRNA, p42-2, targeting from +113 to +121 of human p42 mRNA, was chemically synthesized. On transient transfection p42-2 reduced p42 protein expression ~90% without affecting the expression level of other proteins such as CDK2 (Fig. 2b). Concordantly, in p42-2-transfected cells, CDK2 activity was also inhibited (Fig. 2b). The compromised CDK2 activity was accompanied by a reduction in CDK2 Thr-160 phosphorylation as determined by the anti-CDK2 Thr-160 phospho-specific antibody (Fig. 3b). Moreover, flow cytometry DNA histogram analysis demonstrated an accumulation of HeLa cells in G1 phase of the cell cycle on p42-2 transfection (Fig. 3c). Lack of alteration in abundance and activity of CDK7 on p42-2 transfection excluded the possibility of compromised CDK2 activity as a consequence of a potential p42-CDK7-CDK2 cascade (Fig. 3b). Overall results from both functional interference (dominant-negative) and posttranscriptional gene silencing (siRNA) suggest that p42 is essential for CDK2 activity, at least in cultured cells. One mechanism of the function of p42 on CDK2 activity, as the results also imply, is through Thr-160 phosphorylation of CDK2.

We next examined the ability of p42 to activate CDK2 in vitro. It was shown previously that bacterially produced GST-CDK2 alone is functionally inactive, although it can acquire some histone H1 kinase activity once the Thr-160 is phosphorylated, even in the absence of a cyclin subunit (29). We therefore used this system to test CDK2 activation. GST-CDK2 was first bound to glutathione Sepharose 4B beads and then co-incubated with His6-p42 in the presence of ATP. After co-incubation, His8-p42 was removed by extensive washing whereas GST-CDK2 remained on glutathione Sepharose 4B beads. Finally, the activity of GST-CDK2 before and after co-incubation with His8-p42 was assayed against histone H1 in the presence of [γ-32P]ATP. This test system ensured GST-CDK2 as the only source of measured histone H1 kinase activity. In fact, His8-p42 alone showed no detectable histone H1 kinase activity (data not shown). As a result, without His8-p42 preincubation glutathione bead-bound GST-CDK2 showed no histone H1 kinase activity, in accordance with previous reports (4). Preincubation with His8-p42 significantly enhanced the activity of GST-CDK2, again indicating that p42 activates CDK2 through phosphorylation (Fig. 3d, top left panel).

We then asked whether p42 from tissue culture cells can phosphorylate CDK2 in vitro. p42 was immunoprecipitated from either HeLa cells or HeLa cells ectopically expressing p42. In the latter cells ~3-fold overexpression of p42 was observed (Fig. 2a). As shown in Fig. 3d (top right panel), anti-p42 immunoprecipitates phosphorylate GST-CDK2, and this phosphorylation was eliminated when immunoprecipitation was performed in the presence of competing antigentic peptide. A higher level of GST-CDK2 phosphorylation was detected with p42 immunoprecipitates from HeLa cells overexpressing p42. These results suggest that anti-p42 antibodies immunoprecipitated a kinase directed toward CDK2.

To establish further whether p42 directly phosphorylates CDK2 and whether the phosphorylation occurs specifically on Thr-160, we used purified His8-p42 as the kinase source with GST-CDK2K33R and GST-CDK2T160A as the substrates. Both GST-CDK2 mutants were previously described (4). Using kinase-dead GST-CDK2K33R as a p42 substrate eliminates the remote possibility of CDK2 autophosphorylation in our assays. As a result, His8-p42 phosphorylated GST-CDK2K33R and did not phosphorylate GST-CDK2T160A (Fig. 3d, bottom panel), indicating that p42 is capable of phosphorylating CDK2 on Thr-160 in vitro and that intrinsic kinase activity of CDK2 is not required for this phosphorylation. It is unlikely that the kinase activity was from insect cell-originated contamination because fractions from s9 lysate mock-purified on nickel-aga-
rose beads were not able to phosphorylate GST-CDK2K33R. However, it should be noted that the kinase activity of p42 is weak. Whether the source of p42 was from immunoprecipitates or from baculovirus-produced His6-p42, even under optimized experimental conditions the autoradiographic signal of phosphorylation of GST-CDK2K33R was only visible after prolonged exposure (48 h), whereas the activity of CDK7 CAK immunoprecipitated from the same extracts was easily detected within 2 h of exposure (data not shown). Several reasons may account for this. First, in vitro experimental conditions might not be able to retain the active conformation of p42. Second, p42 activity might require some unknown factors, and when the activation factors are absent or diluted, p42 becomes less active. Third, there might be some inhibitory factors in the cytosol, and when cells are lysed the inhibitory effects on p42 are displayed. Finally, the intrinsic kinase activity of p42 may not be robust both in vivo and in vitro.

To extend the study of the essential role of p42 on CDK2 activity further, we decided to determine how constitutive knockdown of p42 would affect cell growth. For this purpose, a pSuper-based (28) plasmid was constructed that directs the constitutive synthesis of siRNA once transfected into cells. pSuper-p42 was co-transfected into HeLa cells together with pBABEpuro plasmid at a ratio of 10:1, and cells were selected with puromycin. As a result, pSuper-p42-transfected cells generated 10–20-fold fewer puromycin-resistant colonies than pSuper (control plasmid)-

**Fig. 4.** p42 is essential for cell growth. a, constitutive knockdown of p42 impairs clonal growth of HeLa cells. b, expression levels of p42 in stable pSuper and rare surviving pSuper-p42 transfectants. Individual clone numbers are as shown. c, constitutive knockdown of p42 impairs CDK2 activity but not CDC2 activity. CDK2 and CDC2 immunoprecipitation from WCE of the indicated individual clones was performed using anti-CDK2 and anti-CDC2 antibodies. The histone H1 kinase activity associated with the immunoprecipitates was assayed in the presence of γ-32P-ATP. The WCE was also separated on 10% PAGE and immunoblotted with an anti-CDK2 phospho-specific antibody. Individual clone numbers are shown. d, constitutive knockdown of p42 affects cell proliferation. 5 × 10^4 cells of pSuper-p42-4 and -6 or control cells were seeded into 12-well plates. The cell number was counted using the trypan blue exclusion method for 5 continuous days. The growth curves were plotted with means and standard errors representing three independent experiments.
transfected cells (Fig. 4a). Most of the remaining colonies expressed levels of p42 that were indistinguishable from pSuper-transfected cells and probably were not exposed to pSuper-p42 expression (Fig. 4b, data not shown). We conclude that complete inhibition of p42 is probably incompatible with growth or survival of HeLa cells. Interestingly, surviving and nonproliferating pSuper-p42-transfected cells were observed for as long as 2 weeks after the start of puromycin selection (Fig. 4a), arguing against the notion that immediate cell death was the cause of reduction in colony numbers. We were able, however, to recover a few colonies with expression of p42 at ~20–25% of the wild type (Fig. 4b, pSuper-p42 clones 4 and 6). These cells showed significantly slower rates of proliferation in comparison with cells transfected with pSuper (Fig. 4d). It is worth noting that the observed decrease in p42 was not the result of natural clonal variation because p42 was expressed at similar levels among all pSuper-transfected clones.

In addition, pSuper-p42-4 and pSuper-p42-6 clones expressed similar levels of CDK2 as pSuper-transfected clones. However, in pSuper-p42-4 and pSuper-p42-6, CDK2 activity assayed against histone H1 was greatly decreased and accompanied by a reduction in Thr-160 phosphorylation as documented with anti-CDK2 Thr-160 phospho-specific antibody (Fig. 4c). Surprisingly, there was no observable difference of CD2 activity between pSuper and pSuper-p42 clones, implying a potential substrate preference of p42. Similar results were obtained with U2OS p42 knocked down clones (data not shown). Overall, these data suggest that p42 is essential for CDK2 activity and proliferation of human cells.

CDK2 was recently shown to be dispensable for mitotic cell cycles (30–32). This suggests that in addition to CDK2, p42 might phosphorylate other CDKs that are either essential for cell growth or serve as surrogates in CDK2 knock-out cells. Alternatively, this might also point to a possible limitation of knock-out experiments interpretation vis-à-vis the RNA technology for certain issues like the one discussed above and elsewhere (32–35). Specifically, one interpretation would be that inhibition of CDK2 activity during antimitogenic signaling or by other means is different from the loss of CDK2 in knock-out mice with the former causing the cell cycle arrest but the latter failing to do so (33, 36).

In this study we have used a combination of bioinformatic and biochemical approaches to identify a candidate CDK-activating kinase, which we named p42. This protein kinase appears to be essential for CDK2 kinase activity in vivo; when a dominant-negative mutant of p42 was introduced into cells, CDK2 Thr-160 phosphorylation was greatly reduced, and CDK2 activity was compromised. Transient p42 knockdown by siRNA had similar effects. Prolonged knockdown of p42 severely impaired CDK2 activity as well as cell growth. In vitro kinase assays demonstrated that p42 can phosphorylate CDK2 on Thr-160, although the kinase activity of p42 in vitro was rather weak. Nonetheless, the phosphorylation of CDK2 by p42 activated the histone H1 kinase activity of CDK2. We thus conclude that p42 may be a novel mammalian CAK.

Other studies have also suggested the existence of an additional CAK other than CDK7. Kaldis and Solomon (37) biochemically enriched a “small CAK” activity in HeLa cells. The small CAK activity is distinct in size from CDK7 activity; the former peaks at ~30 kDa whereas the latter peaks at ~140 kDa (37). Transforming growth factor β treatment in U2OS cells resulted in low CDK2 Thr-160 phosphorylation without affecting CDK7 activity (27). In D. melanogaster, inactivation of CDK7 homolog with null and temperature-sensitive mutations showed that CDK7 is required for the activation of CDC2-cyclin A and CDC2-cyclin B complexes but not for CDK2-cyclin E complexes (24). It is interesting to note that the additional CAK was frequently linked to CDK2 activation. Indeed, in this study, constitutive p42 knockdown impaired the activation of CDK2 but not CDC2. Moreover, we were not able to establish an appreciable in vitro activity of p42 against CDC2 (data not shown). How such substrate preference arises remains to be determined.

It had been difficult to identify additional mammalian CAK candidates with bioinformatic approaches. The major reason is that the known CAKs share little sequence identity. For example, yeast Cak1p is only 31% identical to that of K. lactis (locus CAS36965), 30% identical to that of Candida albicans (www-sequence.stanford.edu/group/candida), and 20–25% identical to human CDK7. We believed that the candidate mammalian CAK may share only 20–30% identity to yeast Cak1p and, therefore, would blend easily into the background of other protein kinases in the data bases. In this study, we have used a different approach, in which we utilized D. melanogaster as a bridge to link the evolutionary gap between yeast and human. It should be reasonable to assume that homology between budding yeast CAK and D. melanogaster CAK or homology between D. melanogaster CAK and human CAK might be greater than homology between yeast CAK and human CAK. This way we identified a mammalian CAK candidate, and this notion was experimentally supported.

An obvious question is: with a strong CAK (CDK7) why would mammalian cells need another CAK with a lesser activity? We propose several possibilities. First, p42 may have a substrate specificity that is different from CDK7. For example, p42 might prefer CDK2 as the substrate, although this has not been fully documented in this study. Second, because both p42 and CDK7 are nuclear proteins, they might reside in different subnuclear compartments and therefore have different subnuclear accessibility. Third, a two-CAK system may be more flexible than a single CAK system. For instance, one CAK could provide a constitutive housekeeping CAK activity whereas the other might be subject to regulation by growth or other stimuli.

Although p42 was bioinformatically identified here as a potential mammalian Cak1p homolog, it behaves differently from the Cak1p/Csk1 group of CAK. In fission yeast, in which a Cak1p ortholog (Cak1) and a Cdk7 ortholog (Mcs6) co-exist, Csk1 targets both Cdc2 and Mcs6 (21, 23). In this study, p42 activates CDK2 but not CDK7 (Fig. 3, a and b). These discrepancies may suggest evolutionary diversification. After all, grouping CAKs to Cak1p and Cdk7 lineages may be conceptually oversimplified. Recently, Shimotohno et al. (38) identified three novel Cak1p orthologs in Arabidopsis thaliana for a total of four, namely CKA1at, CKA2at, CKA3at, and CKA4at, all of which have different substrate preferences. In single cell budding and fission yeast there is only one cell cycle regulatory CDK (Cdc2/Cdc28), but in mammals there are at least four CDKs directly affecting the cell cycle (Cdc2/Cdc1, Cdk2, Cdk4, and Cdk6). One might not be surprised if more mammalian CAKs(s) have yet to be discovered.

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