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CAK, the p34^{cdc2} activating kinase, contains a protein identical or closely related to p40^{MO15}

Mark J.Solomon[3], J.Wade Harper[1] and John Shuttleworth[2]

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510, [1]Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030, USA and [2]Department of Anatomy, The Medical School, University of Birmingham, Birmingham B15 2TT, UK

[3]Corresponding author

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The mitotic inducer p34^{cdc2} requires association with a cyclin and phosphorylation on Thr161 for its activity as a protein kinase. CAK, the p34^{cdc2} activating kinase, was previously identified as an enzyme necessary for this activating phosphorylation. We confirm here that CAK is a protein kinase and describe its purification over 13 000-fold from *Xenopus* egg extracts. We further show that CAK contains a protein identical or closely related to the previously identified *Xenopus MO15* gene: p40^{MO15} copurifies with CAK, and an antiserum to p40^{MO15} specifically depletes cAK activity. CAK appears to be the only protein in *Xenopus* egg extracts that can activate complexes of either p34^{cdc2} or the closely related protein kinase, p33^{cdk2}, with either cyclin A or cyclin B. The sequence similarity between p40^{MO15} and p34^{cdc2}, and the ~200 kDa size of CAK, suggest that p40^{MO15} may itself be regulated by subunit association and by protein phosphorylations.

Key words: CAK/p34^{cdc2}/p40^{MO15}/protein kinase/p33^{cdk2}

Introduction

A large number of cell cycle transitions are regulated biochemically by protein-protein interactions and by phosphorylations. A paradigm for these controls is the p34^{cdc2} protein kinase, whose activity is required for the G_2 to mitosis transition in all eukaryotic cells (for reviews see Nurse, 1990; Norbury and Nurse, 1992; Solomon, 1993). Monomeric p34^{cdc2} is inactive and unphosphorylated; binding to a cyclin protein is required for both its activity and its phosphorylation (Solomon *et al.*, 1990; Meijer *et al.*, 1991; Parker *et al.*, 1991; Solomon *et al.*, 1992). Three major sites of phosphorylation have been identified, corresponding to Thr14, Tyr15 and Thr161 in the human p34^{cdc2} protein (Gould and Nurse, 1989; Gould *et al.*, 1991; Krek and Nigg, 1991; Norbury *et al.*, 1991; Solomon *et al.*, 1992). Phosphorylation of either Thr14 or Tyr15 is inhibitory; both sites must be dephosphorylated prior to activation. Phosphorylation of Thr161, on the other hand, is absolutely required for p34^{cdc2} activation. Homologs of the *wee1* gene product in *Schizosaccharomyces pombe*, genetically identified as a negative regulator of p34^{cdc2}, phosphorylate Tyr15 (Parker *et al.*, 1992; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993). Thr14 appears to be phosphorylated by a distinct protein kinase (Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993). Homologs of the *S.pombe cdc25* gene product dephosphorylate both Thr14 and Tyr15 (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991; Strausfeld *et al.*, 1991; Lee *et al.*, 1992), thus activating p34^{cdc2}. No strong genetic candidates exist for the protein kinase that phosphorylates Thr161.

An enzymatic activity has been identified in *Xenopus* egg extracts (Solomon *et al.*, 1992) and in mammalian tissue culture cell extracts (Desai *et al.*, 1992) that causes the phosphorylation of p34^{cdc2} on Thr161 in the presence of cyclin. This enzyme was termed CAK, for p34^{cdc2} activating kinase, and was partially purified (Solomon *et al.*, 1992). Phosphorylation of an apparently catalytically inactive form of *Xenopus* p34^{cdc2} (containing a K33R mutation) by partially purified CAK suggested that CAK was a protein kinase, rather than an activator of autophosphorylation by p34^{cdc2} (Solomon *et al.*, 1992). The CAK-induced phosphorylation of p34^{cdc2} was required for its protein kinase activity, but not for its association with cyclin; moreover, Thr161 phosphorylation was not a prerequisite to dephosphorylation on Thr14 or Tyr15 (Solomon *et al.*, 1992). The CAK-induced phosphorylation of Thr161 occurred only in the presence of cyclin, both in a complete *Xenopus* egg extract and in a simplified *in vitro* system using more purified components (Solomon *et al.*, 1992).

Close relatives of p34^{cdc2} have been identified in a number of species and termed 'cdks', for cyclin-dependent protein kinases (Elledge and Spottswood, 1991; Paris *et al.*, 1991; Meyerson *et al.*, 1992); their regulation seems to follow the paradigm established by cyclin-p34^{cdc2}. For instance, p33^{cdk2} can be negatively regulated by phosphorylation on Thr14 and Tyr15, and is positively regulated by phosphorylation on Thr160 (Gu *et al.*, 1992). Although p33^{cdk2} can be dephosphorylated by cdc25 (Gabrielli *et al.*, 1992; Gu *et al.*, 1992) and phosphorylated on Thr160 by CAK *in vitro* (Connell-Crowley *et al.*, 1993), it is not clear if these are the physiologically relevant enzymes for these processes. Just as new and diverged members of the p34^{cdc2} and cyclin families are being discovered, it should not be surprising if more proteins related to wee1, cdc25 and CAK are found, perhaps with shared specificities. Association of cyclins A or B with p34^{cdc2} and p33^{cdk2} may further limit the substrate specificity of these modifying enzymes. Some of these possibilities have been examined in the present work.

We have now enriched CAK over 13 000-fold from *Xenopus* egg extracts, confirmed the previous suggestion that CAK is a *bona fide* protein kinase (Solomon *et al.*, 1992) and shown that p40^{MO15} or a very similar protein is a component of CAK. *MO15* was originally cloned as part of a search for *Xenopus* p34^{cdc2}-related proteins and was

suggested to be a negative regulator of oocyte maturation (Shuttleworth *et al.*, 1990). We show that CAK is the main (or only) activator of both $p34^{cdc2}$ and $p33^{cdk2}$ complexed with either an A- or a B-type cyclin in *Xenopus* egg extracts. Two other groups have reached similar conclusions; their results are reported in the accompanying papers (Fesquet *et al.*, 1993; Poon *et al.*, 1993).

Results

CAK is a protein kinase

It has been suggested that $p34^{cdc2}$ may autophosphorylate on Thr161 (Nigg *et al.*, 1991), since Thr161 aligns with an autophosphorylation site found in a number of protein kinases (Hanks *et al.*, 1988). CAK would then be a stimulator of autophosphorylation by $p34^{cdc2}$, rather than a true protein kinase. Previous work demonstrated that CAK could phosphorylate an apparently inactive mutant of $p34^{cdc2}$ (Solomon *et al.*, 1992). However, since the K33R mutant used in these studies may have a low level of residual protein kinase activity (< ~5% of wild type activity), the possibility that CAK stimulated the autophosphorylation of $p34^{cdc2}$ could not be excluded. The following experiments address this issue more rigorously.

CAK's ATP-binding capability was tested by determining its sensitivity to the ATP analog 5′-*p*-fluorosulfonylbenzoyl-adenosine (FSBA). All protein kinases can bind ATP, whereas a non-kinase activator might not be expected to do so. FSBA inactivates protein kinases by binding to the ATP-binding site and becoming covalently attached to an active site amino acid side chain, expected to be Lys33 in $p34^{cdc2}$ (Zoller *et al.*, 1981; Coleman, 1983). Following incubation, FSBA was inactivated and the residual CAK activity determined in a two part assay: CAK-containing samples were incubated in a kinase buffer with reticulocyte lysate-translated *Xenopus* $p34^{cdc2}$ (Gautier *et al.*, 1991; Solomon *et al.*, 1992) and cyclin B, a bacterially expressed fusion protein containing the enzyme glutathione S-transferase attached to the N-terminus of a sea urchin B-type cyclin (Solomon *et al.*, 1990). The degree of $p34^{cdc2}$ activation was subsequently determined in a direct protein kinase assay, using histone H1 as a substrate. Incubation with FSBA led to a time- and dose-dependent inactivation of CAK (Figure 1a, filled circles and squares). ATP blocked this action of FSBA (open circles and squares), indicating that CAK is indeed an ATP-binding protein.

To determine directly whether CAK possesses protein kinase activity, we examined whether CAK could phosphorylate a kinase-inactive mutant of $p33^{cdk2}$. The human $p33^{cdk2}$ and *Xenopus* $p34^{cdc2}$ proteins are 89% identical, so it was not surprising that CAK could activate $p33^{cdk2}$ in the presence of cyclin (Connell-Crowley *et al.*, 1993). Mutation of a conserved asparagine in the catalytic core of $p33^{cdk2}$ eliminated its kinase activity (<0.01% of wild type activity; Connell-Crowley *et al.*, 1993). This mutant form of human $p33^{cdk2}$ was expressed in *Escherichia coli* and purified to homogeneity (Connell-Crowley *et al.*, 1993). Phosphorylation of the N132A mutant of $p33^{cdk2}$ occurred after incubation with highly purified CAK (Figure 1b) and was greatly stimulated by cyclin B (Figure 1b, compare lanes 8 and 7). Phosphorylation occurred on Thr160 since protein kinase activity toward Thr14 and Tyr15 was separated from CAK at the earliest stages of CAK purification (Solomon *et al.*, 1992). The copurification of the most highly purified CAK with this kinase acting on $p33^{cdk2}$ (Figure 1c) indicates that the two represent the same activity.

Purification of CAK

CAK was purified by conventional means (see Table I) using the activation of $p34^{cdc2}$–cyclin B complexes as an assay. CAK activity was surprisingly stable during this purification. The initial purification of CAK through ammonium sulfate fractionation has already been described (Solomon *et al.*, 1992). Briefly, highly concentrated extracts were prepared (Murray and Kirschner, 1989) from the eggs of 100 frogs that were arrested in interphase following destruction of the endogenous cyclins (Solomon *et al.*, 1990). The extracts were diluted and clarified by ultracentrifugation prior to chromatography on DEAE-Sepharose. Peak fractions were pooled and CAK was precipitated with 38% $(NH_4)_2SO_4$. Further fractionation on hydroxyapatite, Mono Q and

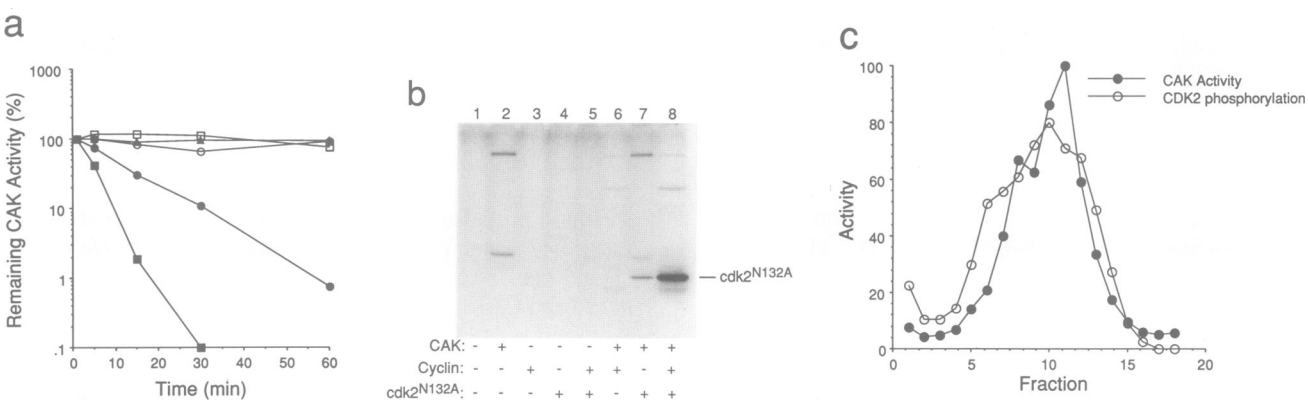

Fig. 1. CAK is a protein kinase. (**a**) Inactivation of CAK by FSBA. Ammonium sulfate-purified CAK was incubated in buffer alone (filled triangles), 0.2 mM FSBA with (open circles) or without (filled circles) added 5 mM ATP or 1 mM FSBA with (open squares) or without (filled squares) added 5 mM ATP, as indicated. The samples were diluted and the FSBA was inactivated by addition of DTT prior to assay of CAK activity. (**b**) CAK directly phosphorylates a kinase-deficient form of $p33^{cdk2}$. Heparin–agarose-purified CAK (fraction 9), GT–cyclin and/or purified $p33^{cdk2}$ protein containing the N132A mutation were added to the indicated incubations in the presence of [γ-^{32}P]ATP. The phosphorylated products were analyzed by gel electrophoresis and autoradiography. (**c**) Copurification of CAK activity and kinase activity toward $p33^{cdk2}$. Fractions from the heparin–agarose column were assayed for CAK activity (same data as in Figure 2b; filled symbols) and for protein kinase activity toward the N132A mutant of human $p33^{cdk2}$ (open symbols). Both activities were quantitated using a PhosphorImager and have been plotted on an arbitrary scale for comparison.

heparin–agarose resulted in an overall purification of 13 100-fold with a yield of 8.9%. The samples in Table I were assayed simultaneously, since quantitative CAK assays are somewhat imprecise and any given level of activity is probably only accurate to within a factor of two (see Materials and methods).

Potential protein components of CAK were examined by comparing the CAK activity profiles across part of the Mono Q column (Figure 2a) and across the heparin–agarose column (Figure 2b) with the Coomassie blue-stained gels of proteins in the same fractions. Major proteins of 41 and 36 kDa copurified with CAK activity. A minor copurifying protein of 43.5 kDa (indicated by the arrow) could be seen in Figure 2b, just above the major 41 kDa protein. This band may represent p40^{MO15} (see below). Partial cDNA clones have been obtained for the 36 kDa and the 41 kDa proteins, using the PCR with degenerate oligonucleotide primers based on the sequences of tryptic fragments of the gel-purified proteins. The sequences of ~50% of the 36 kDa protein and ~26% of the 41 kDa protein have been determined by a combination of protein and DNA sequencing. None of the cloned sequences resembles any reported sequences.

The relationship between CAK and p40^{MO15}

Communication of preliminary experiments that were subsequently reported in one of the accompanying papers (Poon *et al.*, 1993) suggested that CAK might be related to p40^{MO15}. The possibility that CAK contains proteins other than the 41 and 36 kDa proteins was appealing for two reasons. First, neither the cloned 157 amino acids of the 36 kDa protein nor 95 amino acids from sequenced tryptic peptides of the 41 kDa protein showed similarity to any of the conserved motifs indicative of protein kinases (Hanks *et al.*, 1988). Second, one of the tryptic peptides derived from a gel slice containing the 36 kDa protein was identical over its nine residues to a portion of p40^{MO15} (LKPNNLLLD, amino acids 157–165) although this peptide is derived from a highly conserved region in protein kinases (domain VI in Hanks *et al.*, 1998).

To test the possibility that p40^{MO15} was a component of

Table I. Purification of CAK

	Volume (ml)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Fold purification	Yield (%)
Crude lysate	1280	8840	3.98	35 200	1	100
DEAE	1390	828	26.1	21 600	6	61
Ammonium sulfate	22.5	111	147	16 300	37	46
Hydroxyapatite	25.5	10.6	322	3410	81	9.7
Mono Q	2.20	2.39	1100	2620	276	7.4
Heparin–agarose	0.60	0.06	52 300	3130	13 100	8.9

CAK was purified as described in Materials and methods. A CAK concentration of 1 unit/ml is defined as the amount required to activate 30% of the p34^{cdc2} in a standard activation reaction. The calculated yield from this particular assay at the hyroxyapatite step was low. More typically the one step yield from ammonium sulfate through hydroxyapatite was ~50% with a ~5-fold increase in specific activity.

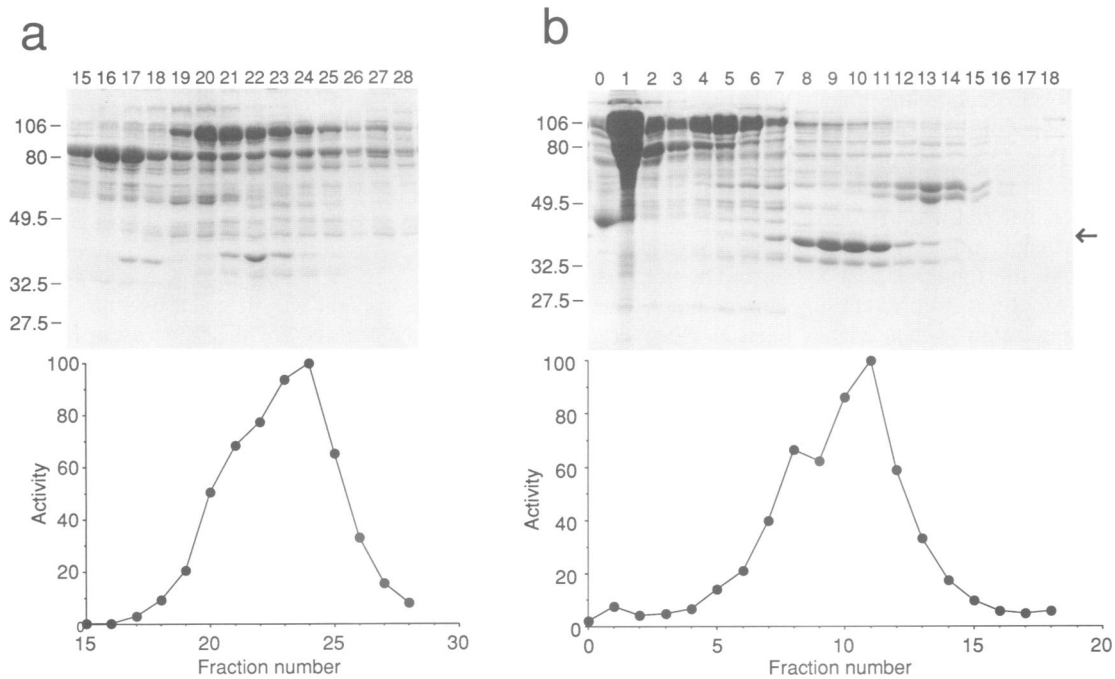

Fig. 2. Purification of CAK. Aliquots of fractions from a portion of the Mono Q column (**a**) and from the heparin–agarose column (**b**) were assayed for CAK activity (graph) or electrophoresed and stained with Coomassie blue (photograph). Fraction 0 in panel b indicates the flow-through material. The samples shown in panel b were electrophoresed on two gels run simultaneously; the splice is located between fractions 7 and 8. Sizes of pre-stained molecular weight markers (Bio-Rad) are indicated in kDa. The arrow in panel b indicates a minor 43.5 kDa protein, just above the major 41 kDa protein, which might correspond to p40^{MO15}.

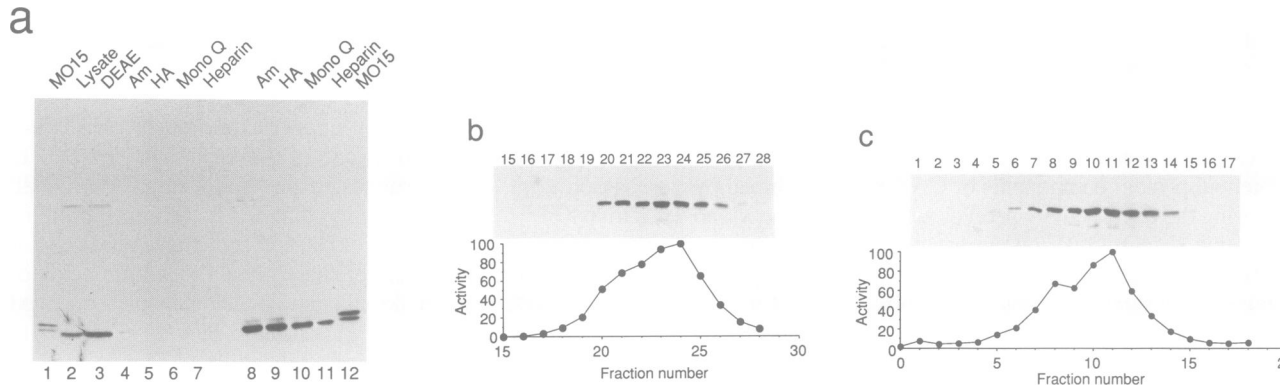

Fig. 3. Copurification of CAK and a p40^{MO15}-like protein. (**a**) Anti-p40^{MO15} immunoblot of samples throughout the CAK purification. Aliquots containing 1 U of CAK activity (lanes 2–7) from the crude lysate (lane 2) or after fractionation on DEAE (lane 3), ammonium sulfate (lane 4), hydroxyapatite (lane 5), Mono Q (lane 6) or heparin–agarose (lane 7), or 5 U of CAK activity (lanes 8–11) after fractionation by ammonium sulfate (lane 8), hydroxyapatite (lane 9), Mono Q (lane 10) or heparin–agarose (lane 11) were electrophoresed and immunoblotted with an antiserum directed against the C-terminal one-third of p40^{MO15}. Lanes 1 and 12 contained 0.5 μl and 1.0 μl, respectively, of a reticulocyte lysate programmed with mRNA for p40^{MO15}; the lower band represents translational initiation at the authentic start site. (**b**) Aliquots from fractions 15–28 of the Mono Q column (lanes 1–14, respectively) were electrophoresed and immunoblotted with anti-p40^{MO15} antiserum. Shown below are the relative CAK activities of these fractions. (**c**) Aliquots from fractions 1–17 of the heparin–agarose column (lanes 1–17, respectively) were electrophoresed and immunoblotted with anti-p40^{MO15} antiserum. Shown below are the relative CAK activities of these fractions.

CAK, we first determined whether it was present in highly purified fractions containing CAK activity (Figure 3a). Samples from each step of the purification were immunoblotted with an antiserum to p40^{MO15} (lanes 2–11). Lanes 1 and 12 contained known amounts of *in vitro*-translated p40^{MO15} and served as size markers and as standards for quantitative estimates of abundance (see Discussion); the upper band of this doublet represents translational initiation at an ATG located 13 codons upstream of the authentic start site (Shuttleworth *et al.*, 1990). Samples containing equal amounts of CAK activity from each purification step were analyzed in lanes 2–7 and samples containing five times as much activity were analyzed in lanes 8–11. A major band of 43 kDa was detected in all fractions, with a mobility almost identical to the lower band of the marker p40^{MO15}. Contaminating bands were seen only at early steps in the purification, even after extended exposures (not shown). This 43 kDa is probably p40^{MO15} itself.

Two conclusions were evident: (i) most of the immunoreactive material was lost during CAK purification and (ii) significant amounts of p40^{MO15}-like material persisted through the final purification steps. The greatest loss of immunoreactivity occurred during ammonium sulfate fractionation (Figure 3a, compare lanes 4 and 3). Recovery from this step onwards was high: comparison of the signal in lane 4 with the signal in lane 8, which contained five times as much material, indicated that the amount of the p40^{MO15}-like protein present after heparin–agarose chromatography (Figure 3a, lane 11) was substantially >20% (probably ~50%) of its level three steps earlier, after ammonium sulfate fractionation (Figure 3a, lane 8). From this and other immunoblots, we estimate that the p40^{MO15}-like protein was enriched ~1300-fold during the ~13 000-fold purification of CAK. This degree of copurification could be fortuitous or could indicate that a physically distinct subset (~10%) of this protein has CAK activity.

The relationship of this p40^{MO15}-like protein to CAK was further analyzed by comparing its distribution with that of CAK following chromatography on Mono Q and heparin–agarose (Figure 3b and c, respectively). The

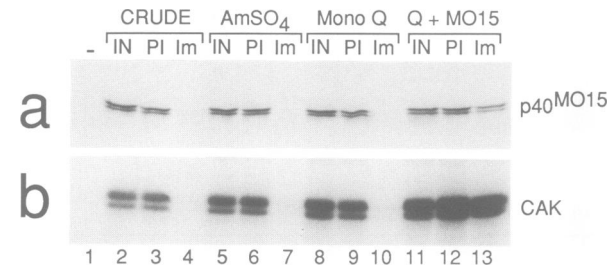

Fig. 4. Immunodepletion of CAK activity by an anti-p40^{MO15} antiserum. Equal amounts of CAK activity from the crude lysate (lanes 2–4), the ammonium sulfate step (lanes 5–7) or from the Mono Q pool (lanes 8–13) were subject to immunoprecipitation. The input material (lanes 2, 5, 8 and 11), supernatant from a precipitation with preimmune serum (lanes 3, 6, 9 and 12), and supernatant from a precipitation with anti-p40^{MO15} antiserum (lanes 4, 7, 10 and 13) were assayed for CAK activity (**b**). (**a**) Shows the efficiency of precipitation of ^{35}S-p40^{MO15} that had been added prior to immunoprecipitation. GST–MO15 was added to the samples in lanes 11–13 to compete specifically for p40^{MO15} binding to the anti-p40^{MO15} antibodies. No CAK was added to the sample in lane 1.

p40^{MO15}-like protein was detected by immunoblotting; the CAK activity profiles are the same as in Figure 2. CAK activity and anti-p40^{MO15} reactivity precisely coeluted. The partial proteolysis of this p40^{MO15}-like protein (Figure 3c) could account for the presence of a p40^{MO15}-like peptide in the 36 kDa region of the gel (see above).

Immunodepletion from crude lysates and extensively purified fractions was used to determine whether the copurifying p40^{MO15}-like protein could account for the observed CAK activity (Figure 4). Antibodies from the preimmune serum and from an anti-p40^{MO15} antiserum were prebound to protein A–agarose beads prior to addition of diluted samples of crude lysate (lanes 2–4), ammonium sulfate-purified CAK (lanes 5–7) or Mono Q-purified CAK (lanes 8–13). A trace amount of ^{35}S-labelled p40^{MO15} from an *in vitro* translation reaction was added to each sample to monitor the efficiency of the immunodepletion (Figure 4a). The input samples (IN), supernatants from the preimmune depletion (PI) and supernatants from the immune

depletion (Im) were assayed for CAK activity (Figure 4b). The preimmune serum failed to deplete any p40MO15 or CAK activity from the three samples (compare lanes 3 and 2, lanes 6 and 5, and lanes 9 and 8). In contrast, the anti-p40MO15 antiserum almost quantitatively depleted both the added ^{35}S-labelled p40MO15 and CAK (compare lanes 4 and 3, lanes 7 and 6, and lanes 10 and 9). Activation of p34cdc2 was reduced to the background level seen in the complete absence of added CAK (Figure 4b, lane 1). A trace of p40MO15 remained in the anti-p40MO15 supernatant from the crude lysate (see below). The ability of added p40MO15 (as a fusion protein to glutathione S-transferase) to block the depletion of both Mono Q purified CAK and the added ^{35}S-labelled p40MO15 verified the specificity of the anti-p40MO15 antiserum (Figure 4, compare lanes 12 and 13 with lanes 9 and 10). Surprisingly, despite the quantitative and specific immunodepletion of the supernatants, only ~5–10% of the input activity was recovered in the precipitates. This poor recovery was due to the inactivation of CAK by even protein A-purified IgG from this antiserum (data not shown).

It is significant that CAK could be immunodepleted from both crude and highly enriched fractions, rather than one or the other alone. Although simple immunodepletion from a crude lysate would also be consistent with a role for a p40MO15-like protein as an activator of CAK, such a function is excluded by immunodepletion from more purified fractions and by the copurification of p40MO15 with CAK activity. Conversely, immunodepletion from only purified fractions would not necessarily indicate that this particular purified activity accounted for the bulk of the activity in crude samples; there could be multiple CAKs. Taken together, these results indicate that a p40MO15-like protein is a component of CAK and accounts for essentially all of the CAK activity in *Xenopus* egg extracts.

We sought additional protein sequence data that could differentiate between p40MO15 and a p40MO15-like protein. We reasoned that the p40MO15-like protein was probably contained at a very low level in the gel slice of the 41 kDa protein submitted for tryptic digestion and HPLC purification of peptides. The sizes of some of these peptides were determined by mass spectrometry. Three out of 14 informative peptides (with masses of 1678, 2941 and 3594) had sizes very close to predicted peptides from p40MO15 (within 0.36%, mean closest match for all 14 peptides 5.44%, median closest match 3.77%). These peptides were considered the best candidates for p40MO15 peptides. The peptide with a 0.36% deviation from a predicted p40MO15 tryptic peptide was sequenced and had a perfect 13/13 match to amino acids 217–229 of p40MO15. We thus consider it very likely that the p40MO15-like protein contained in CAK is actually p40MO15.

CAK activation of p33cdk2

The possibility that CAK is the physiological activator of other members of the p34cdc2 family of protein kinases was tested using purified human p33cdk2 protein as a substrate. Previous work indicated that CAK could activate p33cdk2 in association with either an A- or a B-type cyclin (Connell-Crowley *et al.*, 1993). These studies did not, however, address whether CAK was the only activator of p33cdk2. There could, for instance, be two (or more) enzymes, e.g. 'CAK(cdc2)' and 'CAK(cdk2)', with strong preferences for one substrate, but weak overlapping specificity for the other. Fractions from all four chromatographic separations used

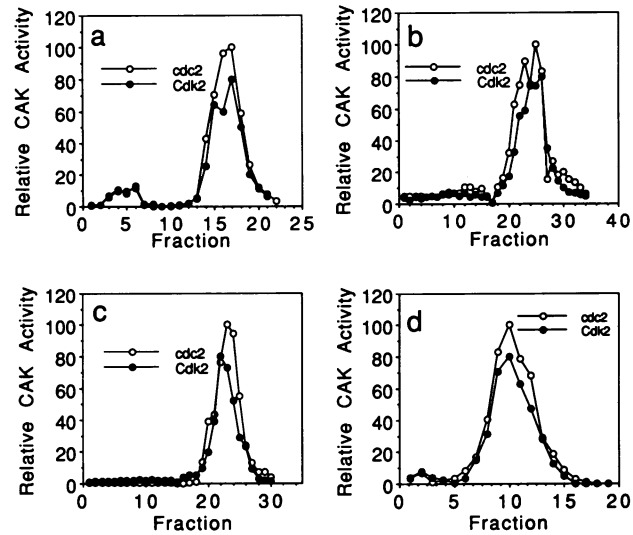

Fig. 5. Copurification of activating kinases for p34cdc2 and for p33cdk2. Fractions from the column chromatography steps in the purification of CAK were assayed for activating kinase activity toward either p34cdc2 (open circles) or p33cdk2 (filled circles) in the presence of cyclin B. Activity was quantitated using a PhosphorImager and has been plotted on an arbitrary scale for comparison. The column steps are DEAE-Sepharose (**a**), hydroxyapatite (**b**), Mono Q (**c**) and heparin–agarose (**d**). Panels c and d represent re-assays of the samples used in Figure 2.

Table II. CAK activates both p34cdc2 and p33cdk2

	CAK(cdc2)[a] (units/ml)	CAK(Cdk2)[a] (units/ml)	Ratio[a]
Crude lysate	27.4	19.3	1.42
DEAE	15.6	5.88	2.65
Ammonium sulfate	726	371	1.96
Hydroxyapatite	134	55.2	2.43
Mono Q	1195	601	1.99
Heparin–agarose	5227	3001	1.74

[a]Aliquots from throughout the purification were assayed quantitatively for their volume specific CAK activity using either p34cdc2 ['CAK(cdc2)'] or p33cdk2 ['CAK(Cdk2)'] as substrate in the presence of cyclin B. 'Ratio' indicates the ratio of 'CAK(cdc2)' activity to 'CAK(cdk2)' activity.

to purify CAK were re-assayed for their ability to activate complexes of cyclin B with either p34cdc2 (Figure 5, open circles) or p33cdk2 (Figure 5, filled circles). 'CAK(cdc2)' and 'CAK(cdk2)' activities were superimposable in all respects including the partial flow-through of activity caused by overloading the DEAE column (Figure 5a). Slight variations in the patterns are insignificant; note, for instance, the small differences between these assays of 'CAK(cdc2)' activity from the Mono Q (Figure 5c) and heparin–agarose (Figure 5d) columns and the previous, independent assays of the identical column fractions (Figure 2a and b). If there are two CAKs in *Xenopus* egg extracts, they either have nearly identical physical properties, or 'CAK(cdk2)' remained bound to one of the columns after elution of 'CAK(cdc2)'.

As an independent test for the presence of a second CAK-like enzyme, we followed the specific activities of CAK toward both p34cdc2 and p33cdk2 at all purification stages (Table II). The ratio of these specific activities remained

relatively constant over the course of the purification, with only some random variation. Given that loss of a 'CAK(cdk2)' by inactivation or strong binding to one of the columns would have increased this ratio dramatically, there appears to be only a single CAK protein.

A third test of whether a single CAK activated all $p34^{cdc2}$- and $p33^{cdk2}$-containing complexes assessed the dependency of kinase activation on the presence of the $p40^{MO15}$-like protein (Figure 6). Immunodepletion supernatants (from Figure 4) from Mono Q-purified CAK (Figure 6a) and from crude lysates (Figure 6b) were tested for their ability to activate complexes of $p34^{cdc2}$ and $p33^{cdk2}$ with either cyclin A or cyclin B. Both cyclins were used in case the specificity of a $p33^{cdk2}$ activating kinase was influenced by the cyclin partner. Immunodepletion of $p40^{MO15}$-like proteins eliminated CAK activity toward all four complexes. For example, immunodepletion from Mono Q-purified CAK reduced CAK activity toward $p34^{cdc2}$–cyclin A from control levels (Figure 6a, compare lane 4 with lanes 2 and 3) to the background level seen in the absence of CAK (Figure 6a, lane 1). The same pattern was observed using the other substrates, although the background activity (in the absence of CAK) of the $p33^{cdk2}$–cyclin A complexes was higher (Figure 6a, lanes 9–12). Similarly, immunodepletion of $p40^{MO15}$-like proteins from the crude lysate eliminated the bulk if not all of the specific activating kinase activity toward all four complexes (Figure 6b). It is difficult to conclude that all of the activity was depleted in this particular experiment since the background was higher (lanes 1, 5, 9 and 13) and since immunodepletion of $p40^{MO15}$ from the crude lysate was incomplete (see above and Figure 4a, lane 4). Some background activation could also be due to the cyclin A-induced partial activation of $p33^{cdk2}$ in the absence of Thr160 phosphorylation (Connell-Crowley *et al.*, 1993).

These three approaches strongly argue that a single CAK protein, containing a protein identical or closely related to $p40^{MO15}$, controls the activation of the four possible complexes of $p34^{cdc2}$ and $p33^{cdk2}$ with cyclin A and cyclin B. We have never seen evidence for a separate enzyme responsible for any significant fraction of the activating kinase activity toward one of these complexes.

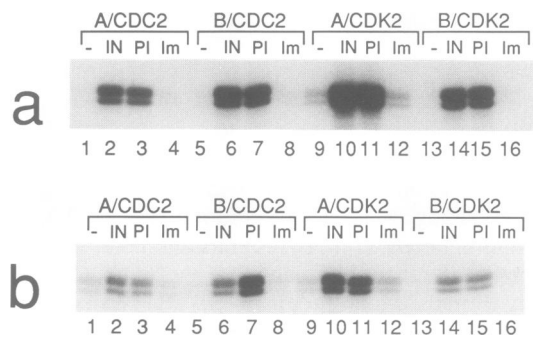

Fig. 6. A $p40^{MO15}$-like protein is required for activation of both $p34^{cdc2}$ and $p33^{cdk2}$ in the presence of either a type A or type B cyclin. $p34^{cdc2}$–cyclin A (lanes 1–4), $p34^{cdc2}$–cyclin B (lanes 5–8), $p33^{cdk2}$–cyclin A (lanes 9–12) and $p33^{cdk2}$–cyclin B (lanes 13–16) complexes were used as substrates for activation by the immunodepleted Mono Q-purified CAK samples (**a**) or the crude lysate samples (**b**) from Figure 4. No CAK (lanes 1, 5, 9 and 13), the input to the precipitations (lanes 2, 6, 10 and 14), the preimmune supernatants (lanes 3, 7, 11 and 15), and the anti-$p40^{MO15}$ immune supernatants (lanes 4, 8, 12 and 16) were assayed, respectively.

Discussion

CAK contains a protein identical or closely related to $p40^{MO15}$

CAK was purified over 13 000-fold on the basis of its ability to activate cyclin–$p34^{cdc2}$ complexes, via phosphorylation of Thr161. Copurification of a $p40^{MO15}$-like protein with CAK activity and immunodepletion of CAK with an antiserum to $p40^{MO15}$ demonstrate that CAK contains either $p40^{MO15}$ itself or a closely related protein. We have used highly enriched fractions to show that CAK is a protein kinase (see also below) and that this single enzyme is responsible for the bulk, if not all, of the activating phoshorylation of both $p34^{cdc2}$ and $p33^{cdk2}$ bound to either cyclin A or B in *Xenopus* egg extracts.

We believe that CAK actually contains $p40^{MO15}$, although neither our data nor that of others (Fesquet *et al.*, 1993; Poon *et al.*, 1993) can completely exclude the possibility that the detected component is actually a protein very closely related to $p40^{MO15}$. This component of CAK shares at least 22 amino acids with *Xenopus* $p40^{MO15}$ and cross-reacts with an antiserum to the C-terminal one-third of $p40^{MO15}$. In addition, only a single protein of the appropriate size was detected in immunoblots of crude lysate proteins; this protein is most likely $p40^{MO15}$.

Among the many $p34^{cdc2}$-like protein kinases to which $p40^{MO15}$ is structurally similar, three stand out. A $p34^{cdc2}$-like gene from rice encodes a protein that is 56% identical to $p40^{MO15}$ over 314 amino acids (Hata, 1991) and, as noted by Poon *et al.* (1993), a probable murine homolog has been identified (Ershler *et al.*, 1993). In yeasts, the most similar sequence is the *Saccharomyces cerevisiae KIN28* gene, which is 45% identical to $p40^{MO15}$ over 334 amino acids and is an essential gene (Simon *et al.*, 1986). Despite the success of genetic approaches in identifying wee1 and cdc25, which were eventually shown to be the protein kinase/phosphatase pair acting on Tyr15 of $p34^{cdc2}$, there exist no strong candidates for CAK. Identification of a functional homolog of $p40^{MO15}$ in *S.cerevisiae* would set the stage for further genetic dissection of the role of CAK in the eukaryotic cell cycle.

Xenopus CAK is rare

Neither of the major proteins in 13 100-fold purified CAK is $p40^{MO15}$. First, the sizes of these proteins are 41 and 36 kDa, whereas the size of the $p40^{MO15}$-like protein detected on immunoblots is 43 kDa. Intriguingly, a faint band at 43.5 kDa copurifies with CAK (Figure 2b) and may represent the $p40^{MO15}$-like protein (see also below). Second, the 41 kDa protein is too abundant to be the $p40^{MO15}$-like protein detected by Western blotting (see below). Third, the sizes of all but an occasional tryptic fragment from the 41 kDa protein (determined by mass spectrometry) were clearly different from the predicted tryptic fragments of $p40^{MO15}$ and the sequences of four tryptic fragments (encompassing 95 amino acids) of the 41 kDa protein showed no similarity to $p40^{MO15}$. Fourth, the single $p40^{MO15}$-like peptide encountered while sequencing tryptic fragments of the 36 kDa protein is probably a contaminant caused by partial proteolysis of the $p40^{MO15}$-like protein. The extremely low sequencing yield of this peptide is consistent with this interpretation. The physiological relationship, if any, of the copurifying 41 and 36 kDa proteins to CAK remains unclear.

It is, however, startling that starfish CAK contains subunits of similar size, 37 and 40 kDa (Fesquet *et al.*, 1993)

Three independent methods were used to estimate the abundance of the p40MO15-like protein in the purified fractions; the results are quite consistent, given their inherent imprecision. (i) The faint 43.5 kDa protein indicated in Figure 2b provides an upper estimate; this protein was present at ~70 ng per lane, or 3.5 ng/μl of each peak fraction. True p40MO15 might be present at a lower, undetectable, level. (ii) Western blotting of purified fractions alongside a known amount of *in vitro*-translated p40MO15 (Figure 3a) provided an independent estimate of ~1 ng/μl in the heparin–agarose eluate. (iii) A minimum estimate is provided by the sequencing of a likely p40MO15-derived tryptic peptide (see Results) that was present at 2.2 pmol. Assuming a sequencing efficiency of 20% (average for all peptides was 10–14%), the heparin–agarose eluate would contain at least 0.85 ng/μl.

Given a p40MO15 abundance of 2–3 ng/μl, we estimate that purification of *Xenopus* CAK to homogeneity would require an overall ~200–400 × 10^3-fold purification from the crude lysate (assuming a heterodimer; see below and Fesquet *et al.*, 1993). The abundance of the p40MO15-like protein in crude lysates, determined by Western blotting (Figure 3a) is ~0.4 ng/μl or one part in ~17 000. This number is in reasonable agreement with the above estimate since only a small subset of the p40MO15-like protein (~5–10%) appears to copurify with CAK. Despite its low abundance, the high turnover number of CAK (see below) would allow it to activate larger amounts of p34cdc2 quite rapidly. It is remarkable that CAK is one to two orders of magnitude more abundant in starfish oocytes (Fesquet *et al.*, 1993).

CAK is an enzyme

CAK has several properties expected of an enzyme. It has a K_m for ATP of ~40 μM, whereas its K_m for p34cdc2 is >100 nM (data not shown). For comparison, p34cdc2 has a K_m for ATP of 75 μM and a K_m for histone H1 of 2000 nM (Erikson and Maller, 1989). More importantly, CAK acts catalytically, rather than stoichiometrically. If the concentration of the 43.5 kDa protein seen to copurify with CAK (Figure 2b) is taken as an upper limit, then each molecule of CAK can phosphorylate and activate at least 40 molecules of p34cdc2 per min, or over 1200 catalytic events in a standard 30 min incubation (this work; Solomon *et al.*, 1992; data not shown). For comparison, the turnover number of human p33cdk2 is ~106 per min at saturating histone H1 concentration (from Connell-Crowley *et al.*, 1993). The true turnover number for CAK is undoubtedly higher, since CAK may be less abundant and since these experiments were performed at p34cdc2 concentrations well below the K_m of CAK.

Since Thr161 aligns with an autophosphorylation site found in some protein kinases, it had been suspected that p34cdc2-like proteins might also autophosphorylate (see for instance Nigg *et al.*, 1991), making CAK an activator of autophosphorylation, rather than a protein kinase. This appears not to be the case. The sensitivity of CAK to FSBA (Figure 1a), and the copurification of CAK with a protein kinase activity (Figure 1c) and with a p40MO15-like protein (Figures 3b, c and 4), indicate that CAK is indeed a protein kinase. The p33cdk2 mutant used here as a protein kinase substrate had <0.01% of wild type activity (Connell-Crowley *et al.*, 1993), a much lower residual level than could be demonstrated for a previously used substrate (Solomon *et al.*, 1992).

It was also reported previously that CAK phosphorylated p34cdc2 in a strictly cyclin-dependent manner. In contrast, Poon *et al.* (1993) and this group observed phosphorylation of p33cdk2 even in the absence of cyclin (Figure 1b). The greater sensitivity of the current assay and the use of p33cdk2 instead of p34cdc2 may have contributed to this difference. Alternatively, subtle misfolding of the bacterially expressed p33cdk2 may have afforded CAK access to Thr160, which it normally acquires only after cyclin binding to p33cdk2. Similar non-physiological phosphorylation of Tyr15 on bacterially expressed p34cdc2 by pp60src has been observed (Gautier *et al.*, 1991).

CAK activation of other CDK proteins

The sequence similarity between p34cdc2 and p33cdk2 suggested that CAK might phosphorylate and activate more than one protein kinase complex. Previous work demonstrated that CAK was capable of activating p33cdk2 in the presence of either cyclin A or B (Connell-Crowley *et al.*, 1993). Our current results indicate that CAK is responsible for the bulk of the activating kinase activity toward all four complexes of p34cdc2 and p33cdk2 with either cyclin A or cyclin B. It remains a remote possibility that 'CAK(cdc2)' and 'CAK(cdk2)', though different, precisely copurify and both contain p40MO15-like proteins. CAK may phosphorylate additional members of the p34cdc2 family of protein kinases. CDK3, like p34cdc2 and p33cdk2, contains a YTHEVV motif surrounding the equivalent of Thr161 within a highly conserved region and is a likely target. An intriguing potential substrate is p40MO15 itself, which contains YTHQVV at the equivalent position: perhaps CAK autoactivates. Other CDK proteins and more distantly related members of this protein kinase subfamily have diverged sequences surrounding this threonine (Meyerson *et al.*, 1992) and may require different activating kinases. Some such complexes may even have a reduced or eliminated dependence on a CAK-like activator (Connell-Crowley *et al.*, 1993). Like p34cdc2 (Meyerson *et al.*, 1992), the cyclins (Xiong and Beach, 1991), cdc25 (Galaktionov and Beach, 1991) and wee1 (Lundgren *et al.*, 1991), there may be a family of CAK-like enzymes, each containing a different p40MO15-related protein.

We suggest that the name CAK be retained for this activity, despite the apparent expansion in its substrate specificity. As it is presently unknown whether CAK can activate all, or only a small subset, of the cyclin-dependent kinases, it would be misleading and possibly erroneous to change the name to one implying a far broader specificity. Moreover, if it becomes necessary, 'CAK' could encompass 'cdk activating kinase' or 'p34cdc2–p33cdk2 activating kinase', as well as 'p34cdc2 activating kinase'.

Implications for the regulation of CAK

There are a number of physical similarities between CAK and the p34cdc2 family of protein kinases; these suggest that some aspects of CAK regulation may follow the p34cdc2 paradigm. Foremost is the 40% identity between p40MO15 and human p34cdc2 (Shuttleworth *et al.*, 1990). The

molecular size of CAK (~200 kDa; Solomon *et al.*, 1992; Fesquet *et al.*, 1993; Poon *et al.*, 1993) suggests that the $p40^{MO15}$-like protein in CAK may also have a binding partner. As with $p34^{cdc2}$, where only a subset of the protein is complexed with cyclin and active (Draetta and Beach, 1988), only a fraction of $p40^{MO15}$ appears to be associated with CAK activity. Indeed, purified starfish CAK contains two proteins, one of them a likely $p40^{MO15}$ homolog (Fesquet *et al.*, 1993). Whether such a binding partner represents a true cyclin, or simply a required regulatory subunit, is not known. Despite these sequence and structural similarities, CAK has no histone H1 kinase activity and $p34^{cdc2}$ has no CAK activity (Solomon *et al.*, 1992).

CAK may also be subject to regulation by phosphorylation(s). As indicated above, $p40^{MO15}$ contains a threonine in a context similar to that of Thr161 in $p34^{cdc2}$. If CAK does not autophosphorylate, there may well be a CAK activating kinase or CAKAK controlling CAK activation. $p40^{MO15}$ does not possess phosphorylatable residues at the equivalent of Thr14 and Tyr15 in $p34^{cdc2}$, and so is not subject to the same form of negative regulation. A key function of these phosphorylations in $p34^{cdc2}$ is to allow accumulation of inactive $p34^{cdc2}$–cyclin complexes prior to their concerted activation at the G_2/M transition (Solomon *et al.*, 1990). Therefore, CAK may be activated gradually, as each molecule of the regulatory subunit binds, rather than abruptly, after a threshold concentration of inactive complexes has accumulated.

The essential nature of Thr161 phosphorylation in $p34^{cdc2}$ activation makes CAK, the enzyme responsible for this phosphorylation, a likely target of regulation during the cell cycle. Thus far, CAK activity appears to be constant during early *Xenopus* development (Solomon *et al.*, 1992). The temporal regulation of the enzymatic activity and substrate preferences of CAK (and CAK family enzymes) have not yet been examined in more complex cell cycles.

Materials and methods

Buffers

Protease inhibitors: 10 μg/ml each of leupeptin, chymostatin and pepstatin (purchased from Chemicon, Temecula, CA). EB: 15 mM $MgCl_2$, 20 mM potassium EGTA, 10 mM DTT, 80 mM potassium β-glycerophosphate, pH 7.3 and protease inhibitors. Buffer D: 10 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, 0.1 mM sodium EDTA, 20 mM triethanolamine (pH 8.0 at 4°C) and protease inhibitors. Buffer HA1: 1 mM $MgCl_2$, 1 mM DTT, 20 mM potassium–PIPES (pH 6.8 at 4°C) and 0.1 × protease inhibitors. Buffer HA2: 1 mM $MgCl_2$, 1 mM DTT, 1 M potassium phosphate (pH 6.8 at 4°C) and 0.1 × protease inhibitors. Buffer H: 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM potassium EDTA, 20 mM potassium–HEPES and protease inhibitors. SDS–PAGE sample buffer: 6.6% SDS, 26% (v/v) glycerol, 100 mM DTT, 262 mM Tris base and bromophenol blue. TB: 25 mM Tris base, 192 mM glycine and 15% (v/v) methanol. TBST: 154 mM NaCl, 10 mM Tris–HCl (pH 8.0 at 23°C) and 0.1% (v/v) Tween 20. IP buffer: EB containing 1 mg/ml ovalbumin and 0.5% NP-40.

Protein reagents

Glutathione S-transferase–cyclin B (GT–cyclin or cyclin B), which contains an N-terminal fusion of the enzyme glutathione S-transferase (Smith and Johnson, 1988) to a sea urchin type B cyclin, was expressed in *E.coli* and affinity purified as previously described by Solomon *et al.* (1990). *Xenopus* $p34^{cdc2}$ protein was expressed in reticulocyte lysates as previously described by Gautier *et al.* (1991). Human $p33^{cdk2}$, the N132A mutant of $p33^{cdk2}$, and human cyclin A were expressed in *E.coli* and purified as previously described by Connell-Crowley *et al.* (1993).

$p40^{MO15}$ was expressed in *E.coli* as a fusion protein with glutathione S-transferase (GST–MO15) and by *in vitro* translation of the cDNA in reticulocyte lysates. Construction of GST–MO15 has been described by Poon *et al.* (1993). The protein was induced as described but was purified according to Solomon *et al.* (1990). For *in vitro* transcription, an SP64T transcription vector containing the MO15 cDNA was linearized with *Eco*RI followed by transcription by SP6 polymerase. The mRNA was translated in 50 μl of a reticulocyte lysate (Promega, Madison, WI) containing 90 μCi of [^{35}S]methionine (Amersham, Arlington Heights, IL), 40 μM cold methionine, or both. TCA precipitation of incorporated [^{35}S]methionine indicated that 9.6 nM $p40^{MO15}$ at a specific activity of 6.1×10^4 c.p.m./μl was synthesized in the absence of added cold methionine and that 11 nM $p40^{MO15}$ was synthesized in its presence.

CAK assays

The CAK assay is a modification of the one described previously by Solomon *et al.* (1992). Briefly, fractions to be assayed were incubated with *Xenopus* $p34^{cdc2}$ and GT–cyclin, diluted and then this reaction was assayed for the histone H1 kinase activity of the activated $p34^{cdc2}$. The use of less $p34^{cdc2}$ in the initial incubation, coupled with a smaller dilution before the histone H1 kinase assay, increased the signal to noise ratio by reducing the low background of activation produced by reticulocyte lysate components in the absence of added CAK. A reaction mix was prepared consisting of 10 μl of reticulocyte lysate programmed with mRNA for *Xenopus* $p34^{cdc2}$ (~12 ng/μl $p34^{cdc2}$), 2 μl of GT–cyclin (~1.9 μM), 2 μl of 1 M $MgCl_2$, 2 μl of 100 mM ATP and 84 μl of EB containing 1 mg/ml ovalbumin. 5 μl of a sample to be assayed for CAK activity were mixed with 5 μl of this reaction mix and incubated at 23°C. After 30 min, 40 μl of EB containing 1 mg/ml ovalbumin were added and the sample was stored on ice or frozen in liquid nitrogen. For the histone H1 kinase assay, 10 μl of the sample were added to 6 μl of a kinase mix containing 0.25 μCi/μl [γ-^{32}P]ATP, 0.267 mg/ml histone H1 [Boehringer Mannheim, Indianapolis, IN; stored in 0.2 M NaCl, 1 mM DTT, 0.1 mM EDTA, 20 mM Tris–HCl (pH 7.4) and protease inhibitors] and 1 mM ATP. The reaction was quenched after 15 min at 23°C by addition of 16 μl of SDS–PAGE sample buffer. Samples were electrophoresed in 10% polyacrylamide gels and subjected to autoradiography.

CAK activation of human $p33^{cdk2}$ complexes (Figure 5) was performed similarly. The reaction mix contained the same components as above, except that the 10 μl of $p34^{cdc2}$ were replaced with 3.3 μl of 2.6 μM $p33^{cdk2}$ protein purified from *E.coli*. GT–cyclin was replaced as necessary by an equal volume of 1.0 μM human cyclin A.

For quantitative CAK assays, the extent of histone H1 phosphorylation was determined by a PhosphorImager scan. A CAK concentration of 1 unit/ml was defined as the concentration necessary to achieve 30% of the maximum possible activation of $p34^{cdc2}$ under the standard conditions using saturating levels of CAK. This level of activation is within the linear range of the assay (data not shown). To ensure consistency and that saturating activation was achieved, specific activities of samples to be compared (see Tables I and II) were always determined simultaneously by assaying dilution series of the samples of interest. Nevertheless, due to the two-step nature of the assay, and the necessity to interpolate from dilution series, there is considerable variability in this quantitative assay. On reassay, the specific activities of individual samples can vary as much as 2-fold.

Direct phosphorylation of the N132A mutant of $p33^{cdk2}$ was performed in a 16 μl reaction containing 3 μl of a 1:100 dilution of heparin–agarose purified CAK, 190 nM $p33^{cdk2}$, 120 nM GT–cyclin, 50 μM ATP and 6.25 μCi [γ-^{32}P]ATP in EB containing 1 mg/ml ovalbumin. Any omitted component (see Figure 1b) was replaced with buffer. SDS–PAGE sample buffer was added after a 30 min incubation at 23°C. For Figure 1c, the reaction contained 3 μl of heparin–agarose purified CAK (1:100 dilution), 300 nM $p33^{cdk2}$, 37 nM GT–cyclin, 35 μM ATP and 3.7 μCi [γ-^{32}P]ATP in a 10 μl reaction.

Purification of CAK

The eggs from 100 frogs were collected and extracts arrested in interphase were prepared as described by Solomon *et al.* (1990). Approximately 120 ml of crude extract was obtained and stored at −80°C until use. Approximately 30 ml of extract were thawed in an ~30°C water bath and diluted with 9 vol of buffer D containing 50 mM NaCl and 2 × protease inhibitors and centrifuged at 55 000 r.p.m. for 45 min in the 60 Ti rotor. The supernatant was loaded at 80 ml/h onto 200 ml of DEAE-Sepharose FF (Sigma, St Louis, MO) equilibrated in buffer D without protease inhibitors in a 2.5 cm diameter column. The column was washed with 450 ml of buffer D containing 50 mM NaCl and proteins were eluted with a 750 ml linear gradient from the same buffer to buffer D containing 300 mM NaCl. Ten millilitre fractions were collected from the beginning of the gradient at a flow rate of 80 ml/h. CAK activity was assayed in undiluted samples and typically peaked in fractions 38–74 (94–142 mM NaCl as determined by conductivity measurements). These fractions were pooled and solid $(NH_4)_2SO_4$ was added to 38%. (Fractionation with $(NH_4)_2SO_4$ was much

sharper when performed after DEAE chromatography, rather than as the first step in the purification.) The precipitate was collected by centrifugation at 20 000 r.p.m. for 20 min in the 35 Ti rotor. The pellets were rinsed once in buffer D containing 50 mM NaCl and 38% $(NH_4)_2SO_4$, resuspended in buffer D containing 50 mM NaCl and dialyzed against 2 l of buffer D containing 50 mM NaCl and 0.1 × protease inhibitors overnight. The precipitate that formed was removed by centrifugation at 10 000 r.p.m. for 10 min in the HB-4 rotor. Samples were quick frozen in liquid nitrogen and stored at −80°C until needed.

The ammonium sulfate fractions from four DEAE column runs were pooled and centrifuged at 50 000 r.p.m. for 10 min in the 60 Ti rotor prior to application onto a TSK hydroxyapatite column (7.5 mm × 7.5 cm; HA-1000, TosoHaas, Philadelphia, PA) using FPLC (Pharmacia). The column was equilibrated in buffer HA1 and run at 1 ml/min. 2.8 ml of sample was applied using a 3 ml loop followed by 5 ml of buffer HA1. A 20 ml gradient was applied beginning at 95% buffer HA1−5% buffer HA2 and ending at 80% buffer HA1−20% buffer HA2. 1 ml fractions were collected beginning at the time of sample application. Seven sequential runs were collected into the same tubes. CAK assays were performed on samples diluted 200-fold into EB containing 1 mg/ml ovalbumin. Fractions 22−24 were pooled and dialyzed overnight against 2 l of buffer D containing 0.1 × protease inhibitors.

The entire sample (25.5 ml) was applied onto a 1 ml Mono Q column (Pharmacia, Pleasant Hill, CA) equilibrated in buffer D containing 20 mM NaCl. The column was rinsed with 2 ml of the same buffer and a 20 ml gradient was applied from buffer D containing 150 mM NaCl to buffer D containing 350 mM NaCl. The column was run at 1 ml/min and 0.5 ml fractions were collected beginning with the 2 ml rinse. Aliquots were diluted 3600-fold into EB containing 1 mg/ml ovalbumin for assay of CAK activity and 20 μl aliquots were added to 20 μl of protein gel sample buffer for SDS−PAGE analysis. Fractions 20−24 were pooled and dialyzed against 1 l of buffer H containing 0.1 × protease inhibitors.

The Mono Q peak of CAK activity was then applied onto a 0.25 ml heparin−agarose column (CNBr linkage, 4% beaded agarose, Sigma) in a 1 ml syringe barrel equilibrated in buffer H. Samples were eluted by a manually applied stepwise gradient. Gradient steps contained 0.5 ml of buffer H followed by 0.1 ml of buffer H containing 180 mM NaCl, 0.1 ml of buffer H containing 200 mM NaCl through 0.1 ml of buffer H containing 480 mM NaCl and 0.5 ml of buffer H containing 500 mM NaCl. Fractions of the same sizes were collected manually. Fraction 0 corresponds to the flow-through material, fraction 1 corresponds to material eluted upon application of buffer H containing 160 mM NaCl and fraction 18 corresponds to the material eluted by buffer H containing 500 mM NaCl. Fractions were diluted 10 000-fold for CAK assays. For protein analysis, 1 μl of 2% (w/v) deoxycholate and 10 μl of 100% (w/v) TCA were added to 88 μl of each fraction. After incubation for 30 min at 4°C, protein precipitates were collected by centrifugation at 14 000 r.p.m. in an Eppendorf microfuge for 10 min at 23°C. The pellets were resuspended in 20 μl of SDS−PAGE sample buffer containing an extra 0.1 M Tris. 4 μl of each precipitated fraction were diluted with 20 μl of 0.5 × SDS−PAGE sample buffer, run on a 10% polyacrylamide gel and stained with Coomassie Blue. The remaining material from the peak of CAK activity (fractions 8−13) was pooled and electrophoresed on two lanes of a 10% polyacrylamide gel. After brief staining and destaining, bands were excised and submitted for protein sequencing of HPLC-purified tryptic fragments by the W.M.Keck Foundation Biotechnology Resource Laboratory (Yale University).

Protein concentrations through the Mono Q column were determined using the Bradford reagent with BSA as a standard. The protein concentration of the heparin−agarose eluate was determined in two ways: (i) the amount of Coomassie blue stainable protein on the gel in Figure 2b was estimated by comparison to known amounts of BSA and (ii) the amount of the 41 kDa protein was determined by amino acid analysis and the total protein in the fractions was assumed to be twice as much. The yield of CAK activity through the purification was 8.9%. This yield would have been ~12.5% if significant aliquots had not been retained at most steps.

5′-p-fluorosulfonylbenzoyladenosine inactivation of CAK

For the time course of FSBA (Sigma) inactivation of CAK, 2 μl of ammonium sulfate-purified CAK were diluted with 18 μl of 50 mM NaCl, 10 mM $MgCl_2$, 1 mg/ml ovalbumin, 10% DMSO and 50 mM potassium−HEPES. Where appropriate, 2 μl of 100 mM ATP were added. Two microlitres of DMSO containing 0, 2 mM or 10 mM FSBA were added at 0 min. At appropriate times, 2 μl were removed and the FSBA was quenched by dilution into 40 μl of EB containing 1 mg/ml ovalbumin prior to determination of CAK activity. CAK activity was quantitated by Cerenkov counting of excised bands.

Immunological methods

Gels for electrophoretic transfer were incubated in TB for 15 min. Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) at 54 V (~420 mA) for 3 h in the Hoeffer (San Francisco, CA) model TE42 Transphor unit. Molecular weight markers were visualized by staining with Ponceau S (Sigma). The membranes were blocked by incubation for 1 h in TBST containing 5% non-fat dry milk and incubated overnight with a 1:500 dilution of anti-$p40^{MO15}$ antiserum (15SSRV, directed against the C-terminal 122 amino acids of $p40^{MO15}$; Shuttleworth *et al.*, 1990) in the same buffer. The filter was rinsed once in the same buffer and then four times for at least 15 min each in TBST. Incubation with an HRP-linked donkey anti-rabbit secondary antibody (Amersham) was performed at a 1:1000 or a 1:10 000 dilution into TBST containing 5% non-fat dry milk. After a 1 h incubation, the filter was washed as above. Detection was by chemiluminescence using the ECL reagents (Amersham).

For immunoprecipitation, 1.0 units of CAK from different stages in the purification was diluted into 200 μl of IP buffer containing 1.0 μl of reticulocyte lysate that had been programmed with mRNA for $p40^{MO15}$ (~9.6 nM $p40^{MO15}$, specific activity of 6.1×10^4 c.p.m. [^{35}S]$p40^{MO15}$/μl). For each precipitation, 15 μl of protein A−agarose beads were washed with IP buffer, incubated in IP buffer with 5 μl of immune or preimmune antiserum for 1 h with rotation to pre-bind the IgG, washed with IP buffer and incubated with the sample for immunoprecipitation. Preliminary experiments indicated that 5 μl of antiserum was the minimum necessary for quantitative immunodepletion under these conditions. After 1 h with rotation, the beads were pelleted, washed 2−4 times in IP buffer and resuspended in 200 μl of IP buffer. 5 μl aliquots were removed for CAK assay and 10 μl aliquots were removed for electrophoretic/autoradiographic analysis of the efficiency of immunoprecipitation. Addition of ~0.3 μg GST−MO15 was used to block specific precipitation in some samples.

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