The *MO15* gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues

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Phosphorylation of Thr161, a residue conserved in all members of the cdc2 family, has been reported to be absolutely required for the catalytic activity of cdc2, the major regulator of eukaryotic cell cycle. In the present work, we have purified from starfish oocytes a kinase that specifically activates cdc2 in a cyclin-dependent manner through phosphorylation of its Thr161 residue. Our most highly purified preparation contained only two major proteins of apparent M_r 37 and 40 kDa (p37 and p40), which could not be separated from each other without loss of activity. The purified kinase was found to phosphorylate not only cdc2, but also cdk2 and a divergent cdc2-like protein from *Caenorhabditis*, in chimeric complexes including both mitotic and G_1/S cyclins. Extensive microsequencing of p40 did not reveal any convincing homology with any known protein. In contrast, p37 is the starfish homologue of the *MO15* gene product, a kinase previously cloned by homology probing from a *Xenopus* cDNA library. As expected, immuno-depletion of the MO15 protein depleted *Xenopus* egg extracts of CAK (*cdk-activating kinase*) activity, which was recovered in immunoprecipitates. Taken together, the above results demonstrate that *MO15* is a gene conserved throughout evolution (at least from echinoderms to vertebrates) that encodes the catalytic subunit of a protein kinase that activates cdc2–cdks complexes through phosphorylation of Thr161 (or its homologues).
Key words: cdc2/cell cycle/cyclin-dependent kinases/enzyme purification

Introduction

Transitions of the cell cycle are controlled in all eukaryotes by oscillations in the activity of kinase complexes between cdc2 or cdc2-like proteins and cyclins. The first of these complexes to be identified was MPF (M phase promoting factor), which universally controls entry into and exit from M phase (for reviews see Dorée, 1990; Nurse, 1990; Masui, 1992). Biochemical purification of MPF to near homogeneity in several species has firmly established that it consists of an heterodimeric complex between one molecule of cdc2 and one molecule of cyclin B (Labbé *et al.*, 1989; Gautier *et al.*, 1990; Kusubata *et al.*, 1992; Yamashita *et al.*, 1992).

cdc2 (CDC28 in budding yeast) is the catalytic subunit of a protein kinase first demonstrated by genetic analysis in fission yeast to control the G_2 to M phase transition (reviewed in Nurse, 1985). Mitotic cyclins are a family of proteins, first discovered in embryos of marine invertebrates, whose abundance follows a sawtooth pattern throughout each cell cycle, due to their accumulation then sudden degradation at the metaphase to anaphase transition (reviewed in Hunt, 1989). This behaviour actually characterizes two sub-families, cyclins A and B, which can be distinguished by specific motifs that are conserved throughout evolution (Nugent *et al.*, 1991). Cyclin B synthesis and degradation are both necessary and sufficient to maintain in cell-free extracts prepared from early *Xenopus* embryos the alternation between S and M phase (Minshull *et al.*, 1989; Murray and Kirschner, 1989; Murray *et al.*, 1989). Cyclin A appears to play multiple roles during the cell cycle and has been reported to be required for DNA replication (Girard *et al.*, 1991; Pagano *et al.*, 1992; Zindy *et al.*, 1992), to maintain dependence of mitosis on completion of DNA replication (Walker and Maller, 1991) and to potentiate MPF activation (Devault *et al.*, 1991, 1992).

While in both fission and budding yeast another major control point of the cell cycle, the G_1/S transition, is controlled through association of a unique catalytic subunit, cdc2/CDC28, with different types of cyclins, including G_1 cyclins (CLN-type) (reviewed in Reed, 1992), a family of cdc2-related proteins (called cdks) mediate this function in vertebrates through association with a variety of cyclins classified as C, D and E (Koff *et al.*, 1991; Leopold and O'Farrell, 1991; Lew *et al.*, 1991; Matsushime *et al.*, 1991, 1992; Motokura *et al.*, 1991; Paris *et al.*, 1991; Tsai *et al.*, 1991; Xiong *et al.*, 1991; Inaba *et al.*, 1992; Meyerson *et al.*, 1992; Okuda *et al.*, 1992; Rosenblatt *et al.*, 1992).

Changes in the kinase activity of cdc2–cdks depend not only on their association with a variety of cyclins but also on post-translational modifications by phosphorylation–dephosphorylation reactions. Not surprisingly, changes in the activity of MPF were first analysed. Inhibitory phosphorylations that delay MPF activation until correct DNA replication has been completed were first discovered (for review see Murray, 1992). The main inhibitory phosphorylation is that of Tyr15 (Gould and Nurse, 1989), with a further inhibitory phosphorylation on Thr14 also occurring in higher eukaryotes (Solomon *et al.*, 1990; Krek and Nigg, 1991; Norbury *et al.*, 1991). The main protein kinase responsible for Tyr15 phosphorylation is wee1, the product of a conserved gene first discovered in fission yeast as negatively controlling the G_2 to M phase transition (Russell and Nurse, 1987; Honda *et al.*, 1992; Parker *et al.*, 1992; McGowan and Russell, 1993). The main phosphatase antagonistic to wee1 and responsible for dephosphorylation of Tyr15 (perhaps also Thr14) is cdc25, the product of a conserved gene first discovered as positively controlling the G_2 to M phase transition in fission yeast (Russell and

Nurse, 1986; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Strausfeld *et al.*, 1991). However, phosphorylation – dephosphorylation reactions of cdc2 on Tyr15 and Thr14 are not essential features of the eukaryotic cell cycle. Indeed, some cycles (for example the first 12 embryonic cell cycles with exception of the first one in *Xenopus*: Ferrell *et al.*, 1991) occur in the absence of such events. Moreover, feedback mechanisms unrelated to Tyr15 phosphorylation prevent entry into mitosis before completion of DNA replication, at least in some organisms (Amon *et al.*, 1992; Sorger and Murray, 1992).

In contrast, another phosphorylation of cdc2 that appears to be absolutely required for its catalytic activity occurs at Thr161 (Thr167 in fission yeast), a residue that is conserved in all members of the cdc2 family. In fission yeast, thermosensitive (ts) mutants of *cdc2* cannot be rescued by ectopic expression of *cdc2* mutated at Thr167 (Ducommun *et al.*, 1991; Gould *et al.*, 1991) with the possible exception of cdc2-E 167 (see below). In somatic cells of vertebrates as well as in cell-free systems derived from *Xenopus* eggs, formation of cyclin B – cdc2 complexes is correlated with Thr161 phosphorylation (Krek and Nigg, 1991; Solomon *et al.*, 1992). This has been extended to complexes of cyclin A with either cdc2 or cdk2 (Devault *et al.*, 1992; Gu *et al.*, 1992). Inactivation of cyclin A – cdc2 and cyclin B – cdc2 complexes at the metaphase to anaphase transition is associated with both cyclin degradation and Thr161 dephosphorylation (Lorca *et al.*, 1992a). Moreover, ts *cdc2* mutants of fission yeast that overexpress cdc2-E167 accumulate at mitosis, suggesting that E167 mimics constitutive phosphorylation of Thr167 and that this residue of cdc2 must be dephosphorylated for cells to exit from mitosis (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Krek *et al.*, 1992).

Although Thr161/167 is localized in the so-called 'autophosphorylation' domain of serine/threonine kinases (Hanks *et al.*, 1988), it is not possible to transfer ^{32}P from [γ-^{32}P]ATP to Thr161 in highly purified cyclin B – cdc2 kinase (Labbé *et al.*, 1991). Moreover a kinase activity distinct of cdc2 and able to phosphorylate Thr161 in cdc2 has been partially purified from *Xenopus* eggs (Solomon *et al.*, 1992). This kinase may be essential to confer its catalytic activity to cdc2 – cdks complexes. In the present work, we extensively purified from starfish oocytes a kinase that specifically phosphorylates and activates cdc2 and cdc2-like proteins in a cyclin-dependent manner. We partially sequenced the two polypeptides that remain associated at the final step of our purification procedure and found that one of these polypeptides is the starfish homologue of *Xenopus* p40^{MO15}, a cdc2-related protein kinase previously isolated from a cDNA library using oligonucleotides with sequences deduced from the conserved catalytic domains of serine/threonine kinases (Shuttleworth *et al.*, 1990). The second polypeptide is unknown, but it may be a regulatory subunit required to confer to the MO15 protein its catalytic activity. Another group reached independently similar conclusions by showing that bacterially expressed MO15 protein acquires the ability to phosphorylate cdk2 at Thr160 (the equivalent of Thr161 in cdc2) after incubation with cell-free extracts prepared from *Xenopus* eggs. Their results are described in the accompanying paper (Poon *et al.*, 1993).

Results

An in vitro assay for production of active cdc2 kinase in extracts prepared from starfish oocytes

Addition of bacterially expressed human cyclin A to extracts prepared at interphase from activated *Xenopus* eggs induces the formation of an active H1 kinase complex between cyclin A and cdc2 without a lag phase, and to a minor extent with cdk2 (Clarke *et al.*, 1992). We showed previously that formation of this major complex is associated with phosphorylation of a unique threonine residue in cdc2 and mapped this residue to Thr161. We also reported that this phosphorylation paradoxically increases mobility of cdc2 (Devault *et al.*, 1992; Lorca *et al.*, 1992a). Phosphorylation of Thr161 is probably due to a specific kinase that has been partially purified by others from frog extracts (Solomon *et al.*, 1992).

When the same recombinant cyclin A was mixed with *Xenopus* cdc2, translated *in vitro* in reticulocyte lysate or immunoprecipitated from interphase extracts, only a very small amount of H1 kinase activity (if any) was produced (Figure 1a) and no change of cdc2 electrophoretic mobility was detected (Figure 1b). In contrast, a high H1 kinase activity was produced when an extract prepared from starfish oocytes was further added, even when this extract was first purified by affinity chromatography on p13^{suc1} – Sepharose beads to deplete cdc2 and cdc2-like proteins (Figure 1a). Formation of an active kinase complex was associated with an increased electrophoretic mobility of cdc2 (Figure 1b) and when [γ-^{32}P]ATP was added to the extract, ^{32}P was found to be incorporated into phosphothreonine (Figure 1c). These preliminary results suggested that the starfish oocyte extract contained a kinase activity that phosphorylates cdc2 on Thr161 in a cyclin-dependent manner. By analogy to the frog kinase, we thereafter refer to the starfish kinase as to CAK (previously defined as *cdc2-a*ctivating *k*inase).

Purification of starfish CAK

We have purified starfish CAK on the basis of its ability to activate *Xenopus* cdc2 in a cyclin-dependent manner. The starting material was an extract prepared from the oocytes of the starfish *Marthasterias glacialis* because it is easy to obtain large amounts of such oocytes, all are similar in size and arrested at first meiotic prophase and because CAK activity does not appear to increase when such oocytes are released from meiotic arrest by hormonal stimulation. In brief (Figure 2) a high speed supernatant was applied to a sulphopropyl (SP)-Sepharose fast flow column and eluted with a NaCl gradient (panel a). The pooled fractions of the unique peak of activity were loaded onto a heparin – Sepharose column and eluted with a NaCl gradient (panel b). Further purification was obtained by gel filtration on a Superdex 200 column (panel c). Positive fractions were pooled and fractionated on a Mono Q column (panel d). Finally, maximal purification was achieved by loading the pooled peak of activity on a Mono S column that was eluted by a NaCl gradient (panel e). Figure 3 depicts the pattern of proteins associated with the successive peaks of CAK activity after separation by electrophoresis on a polyacrylamide gel under denaturing conditions. Due to the relative complexity of the two-step assay, it is difficult to evaluate
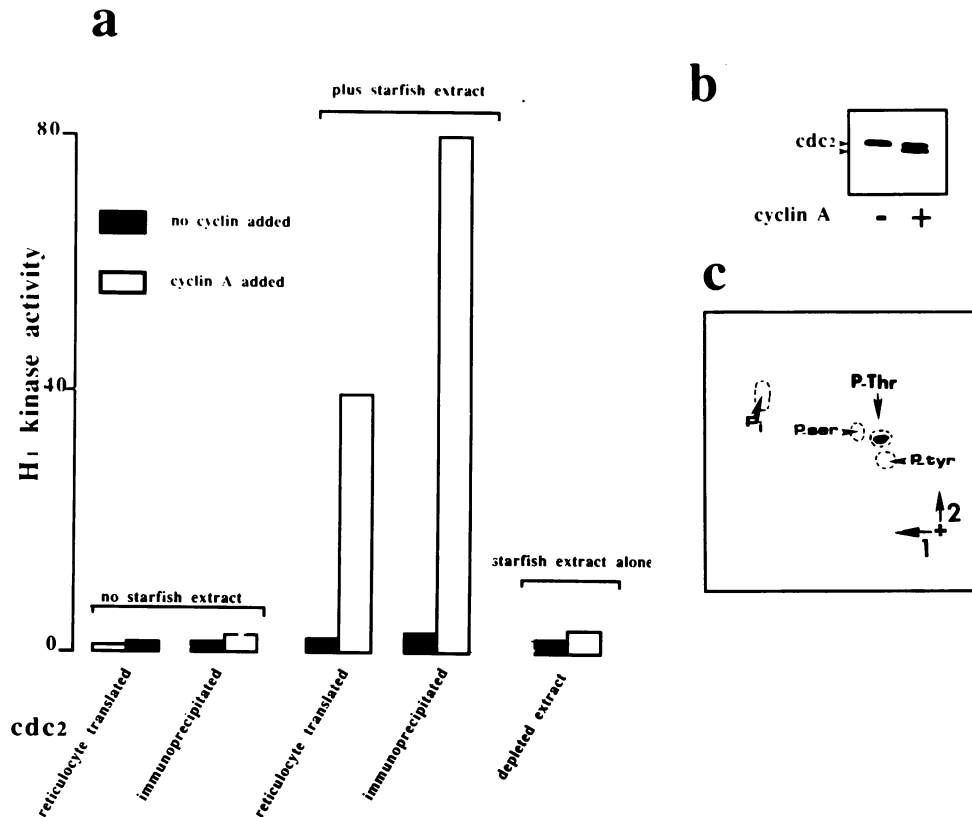
Fig. 1. Starfish oocyte extracts contain a cdc2-activating kinase that phosphorylates cdc2 in a cyclin-dependent manner. (a) *Xenopus* cdc2, translated from the corresponding mRNA in reticulocyte lysate or immunoprecipitated from interphase extracts, was assayed for H1 kinase activity in the presence or absence of added human cyclin A (0.1 μg/μl), produced in *E.coli* (the H1 kinase activity measured in reticulocyte lysate without cyclin A addition is taken as unit). An extract prepared from starfish oocytes at first meiotic prophase (see Materials and methods) was first depleted of its basal H1 kinase activity by affinity chromatography on p13*suc1* beads and added or not to *in vitro* translated or immunoprecipitated cdc2. H1 kinase activity was assayed 15 min later. (b) Same experiment as in panel a, but *Xenopus* cdc2 was translated *in vitro* in the presence of [35S]methionine and immunoprecipitated after 15 min incubation with starfish homogenate in the presence (+) or absence (−) of cyclin A. Immunoprecipitated cdc2 was run on a 12.5% polyacrylamide gel in the presence of SDS and the dried gel submitted to fluorography. (c) Same experiment as in panel a, but [γ-32P]ATP was added (10 μCi/50 μl) to the starfish homogenate before its addition (with cyclin A) to immunoprecipitated cdc2. After 15 min the immunoprecipitate was solubilized and cdc2 recovered by SDS−PAGE and analysed, after electroblotting and hydrolysis, by autoradiography for phosphoamino acid content (see Materials and methods).

precisely the extent of CAK purification. We estimate that CAK was purified >500-fold and with a recovery of ~8% from SP-Sepharose to the final step (Table I) and at least 10 times more from the initial homogenate.

The final preparation that we routinely obtained using the above procedure was completely free of any H1 kinase activity and contained only three major polypeptides of apparent M_r 110, 40 and 37 kDa (Figure 3, lane 7). Although the size of native CAK was estimated to be ~220 kDa by gel filtration on the Superdex column, suggesting a possible 1:1:1 subunit structure, side by side analysis of successive fractions across the Mono Q peak of activity showed that the 110 kDa polypeptide did not peak with CAK activity (Figure 4). Moreover, the high molecular weight polypeptide was almost quantitatively eliminated from the final preparation when a single fraction was taken from each peak of activity instead of the pooled active fractions and applied to the next column (Figure 5). Thus the 110 kDa polypeptide is a contaminant, not a component of starfish CAK.

In contrast, both the 40 and the 37 kDa polypeptides peaked exactly with CAK activity during the last steps of

the purification (Figure 4). Moreover, we failed in all further attempts to separate them from each other using hydrophobic chromatography on phenyl Superose, gel filtration at high ionic strength (2 M NaCl) or chromatography on Cibacron blue (not shown). These results strongly suggest that starfish CAK is an heteromeric complex between two components of apparent M_r 40 and 37 kDa (p40 and p37). Their exact stoichiometry in the active protein kinase complex has not yet been determined.

The p40 and p37 polypeptides were then electroblotted onto nitrocellulose and digested *in situ* with trypsin. The released peptides were separated and part of them sequenced. Although seven different peptides derived from p40 were sequenced, including a total of 136 amino acids, no significant homology was found with any known protein in available data bases (data not shown).

In contrast microsequencing of six different peptides (86 amino acids) derived from p37 revealed a high degree of homology (80% of identity and 94% similarity) with the product of *MO15* (Figure 6). Polyclonal antibodies directed against a fusion protein comprising the C-terminal 122 amino acids of MO15 actually recognized starfish p37 by Western

blotting, although they failed to immunoprecipitate CAK activity (not shown). This led us to investigate whether *Xenopus* MO15 protein (*Xenopus laevis* p40^{MO15}) was also associated with CAK activity. As shown on Figure 7a, antibodies against *X.laevis* p40^{MO15} readily depleted interphase *Xenopus* extracts from both p40^{MO15} and CAK activity (compare bars 1 and 2) and CAK activity was recovered in immunoprecipitates (bar 3). We also found that both CAK activity and *X.laevis* p40^{MO15} are associated with the prominent nucleus known as germinal vesicle (GV) in prophase-arrested oocytes (Figure 7b). This may explain why microinjection of human cyclin A generates chimeric

cyclin A–cdc2 kinase activity much more rapidly in nucleated than enucleated *Xenopus* oocytes (Figure 7c).

Nonetheless, identification of the catalytic subunit of CAK as the product of the *MO15* gene came as a surprise because MO15 was previously reported to delay cdc2 kinase activation by progesterone. An opposite role would rather have been expected for CAK. Perhaps the MO15 protein plays different roles, depending on post-translational modifications and/or its association with other proteins. As a matter of fact, both high mobility and low mobility forms of the protein were detected and only a small part of *X.laevis* p40^{MO15} ($\sim 15\%$) was found to be engaged in a high
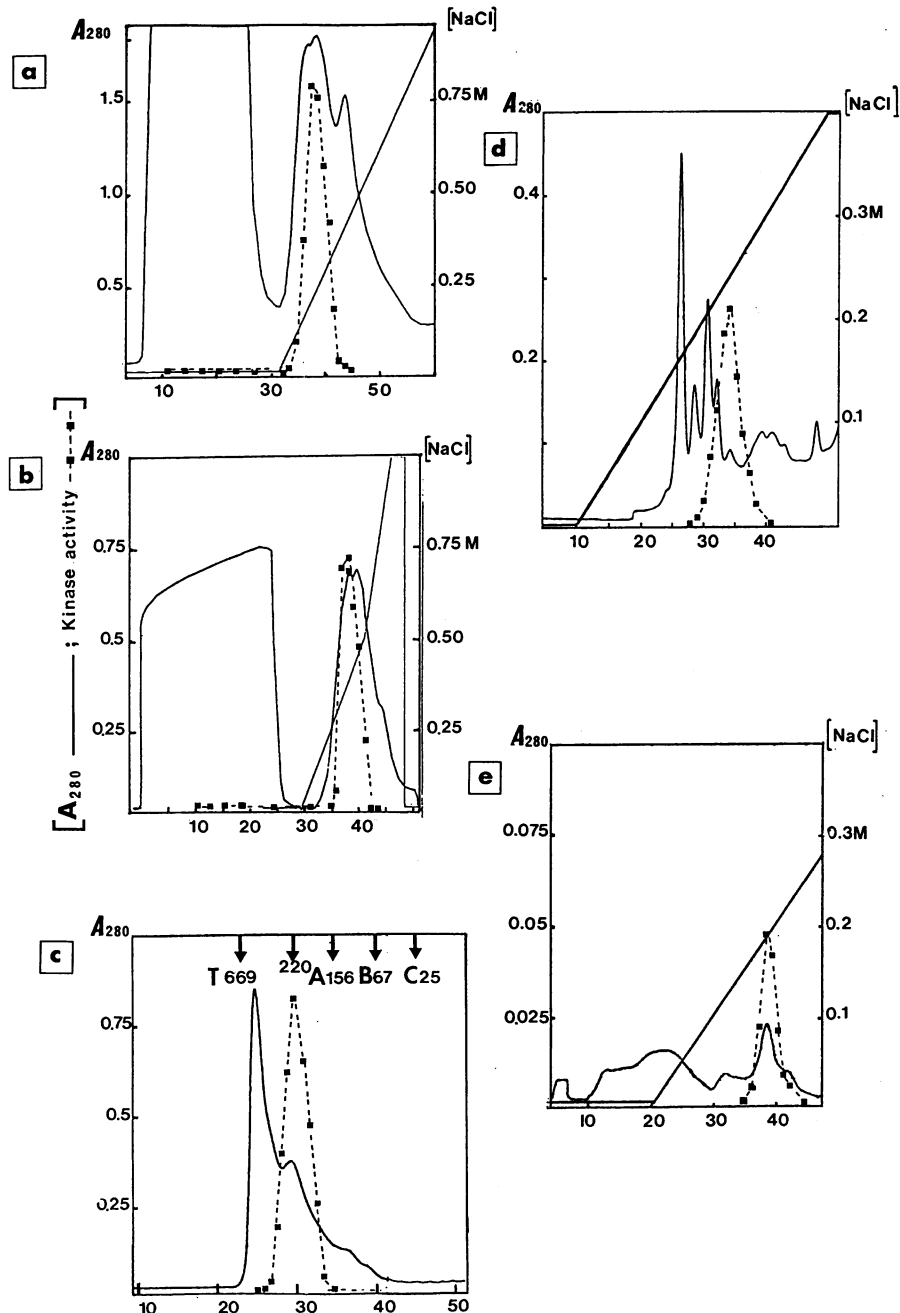

Fig. 2. Purification of starfish CAK along successive steps of column chromatography. Optical density at 280 nm was recorded at each step of the purification (solid line) and successive fractions assayed for CAK activity (dashed line). (**a**) Chromatography on SP-Sepharose; (**b**) chromatography on heparin–Sepharose; (**c**) chromatography on S200 Superdex: elution volumes of thyroglobulin (T, 669 kDa), aldolase (A, 156 kDa), bovine serum albumin (B, 67 kDa) and chymotrypsinogen (C, 25 kDa) are shown by arrows, as well as the elution volume of CAK activity (220 kDa); (**d**) chromatography on Mono-Q; (**e**) chromatography on Mono-S. See Materials and methods for a complete description of the fractionation procedure.

molecular weight complex of high CAK activity (Figure 8) when an interphase *Xenopus* extract was fractionated by gel filtration on a Superdex 200 column.

Properties of purified starfish CAK

Starfish CAK phosphorylates Thr161 in cdc2. The finding that phosphorylation of a threonine residue of cdc2 by starfish CAK both increases its electrophoretic mobility and confers cdc2 its H1 kinase activity, strongly suggested that CAK phosphorylates Thr161. Two approaches were used to confirm this view. First, mutants of cdc2 were translated in reticulocyte lysates and used as putative substrates for purified CAK. As shown on Figure 9, substitution of Thr14 and Tyr15 with non-phosphorylatable residues did not affect the ability of starfish CAK to phosphorylate and activate cdc2. In contrast, substitution of Thr161 with alanine completely abolished phosphorylation and activation of cdc2. These results show that integrity of Thr161 is required for cdc2 to undergo phosphorylation and activation by CAK, but do not formally demonstrate that CAK phosphorylates Thr161 (Thr161 could as well be required for CAK to bind cdc2 and this event itself required for CAK to phosphorylate cdc2 elsewhere). To provide direct evidence for actual

Fig. 3. Analysis by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining of the pattern of proteins in active fractions along starfish CAK purification. Lane 1, molecular weight markers; lane 2, crude homogenate; lane 3, SP-Sepharose step; lane 4, heparin–Sepharose step; lane 5, Superdex 200 step; lane 6, Mono Q step; lane 7, Mono S step; lanes 2–6, an aliquot of the pooled peak fractions was loaded; lane 7, an aliquot of the peak (fraction 39, see Figure 2e) was loaded.

phosphorylation of Thr161, cdc2 was phosphorylated by CAK in the presence of $[\gamma\text{-}^{32}P]$ATP. Three ^{32}P-labelled fragments of 28, 25 and 21 kDa were produced when cdc2 was degraded by *N*-chlorosuccinimide (Figure 10a). This corresponds to the size of *N*-chlorosuccinimide-generated fragments when cdc2 undergoes phosphorylation on Thr161 in interphase *Xenopus* extracts (Lorca *et al.*, 1992a). Moreover, a single labelled peptide was produced when cdc2 was digested with trypsin (Figure 10b) or successively with trypsin and the V8 protease (Figure 10c). This last peptide was found to migrate exactly as a synthetic Val-Tyr-Thr(P)-His-Glu peptide, which corresponds to the phosphorylated 159–163 fragment of *Xenopus* cdc2.

Starfish CAK is a specific kinase that uses both ATP and GTP to phosphorylate cdc2 and cdc2-like proteins in a cyclin-dependent manner. We tried to phosphorylate proteins that are substrates for a variety of protein kinases with starfish CAK. This includes mixed histones, casein, myelin basic protein, phosphorylase b, tubulin or phosvitin. None of these proteins was a substrate for starfish CAK. The purified kinase also failed to phosphorylate several synthetic peptides containing the sequence that spans Thr161 in cdc2 and its homologues, in the absence as well as in the presence of added cyclin (data not shown).

In contrast starfish CAK readily phosphorylated and activated *Xenopus* cdk2 in the presence of cyclin A (Figures 11 and 13). GTP could be used as effectively as ATP to phosphorylate and activate either cdc2 or cdk2 immunoprecipitated from *Xenopus* extracts with specific antibodies raised against the C-terminal tail of these proteins (not shown).

The *ncc-1* gene, isolated from a *Caenorhabditis* cDNA library by H.Mori and P.Sternberg, was recently sequenced in our laboratory (C.Ferraz, unpublished data; EMBL data bank, accession number X68384). The protein predicted to be encoded by this gene is an homologue that differs from cdc2 by the unusual substitution of valine for isoleucine in the highly conserved 'PSTAIRE' box of cdc2 homologues, as well as by N-terminal and C-terminal extensions of 14 and nine amino acids, respectively. As shown on Figure 11, starfish CAK was found to phosphorylate and activate this divergent cdc2 homologue in a cyclin-dependent manner. Although we did not use any other homologue as putative substrate, the above results strongly suggest that CAK may phosphorylate and confer kinase activity to the increasing number of cdc2-like proteins reported to occur in multicellular organisms (Meyerson *et al.*, 1992; Okuda *et al.*, 1992).
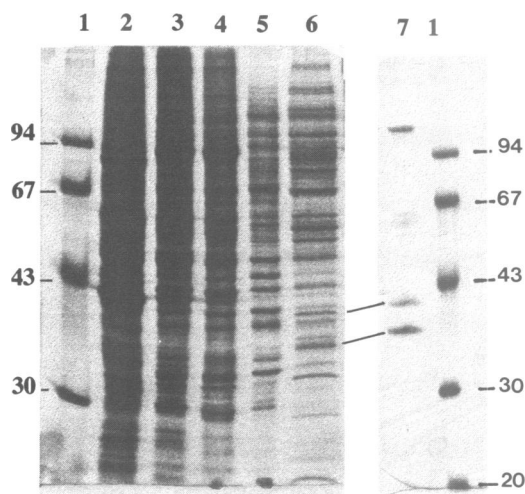
Table I. Purification of starfish CAK

Step	Volume (ml)	Total proteins (mg)	Specific activity[a] (units/mg)	Total activity (units)	Yield[b] (%)
SP-Sepharose	60	510	1	510	100
Heparin–Sepharose	27	80	2.9	232	45
Superdex 200	6	9	5.7	51.3	10
Mono Q	1.2	0.45	113	50.9	10
Mono S	1.6	0.075	544	40.8	8

[a]Relative CAK activity at any step of the purification was estimated from the dilution that causes half-maximal cyclin A-dependent activation of reticulocyte-translated cdc2 in our experimental conditions. Specific activity of the pooled peak fractions eluted from the SP-Sepharose column is arbitrarily taken as a unit.
[b]CAK activity recovered after the SP-Sepharose step is arbitrarily taken as 100% yield.
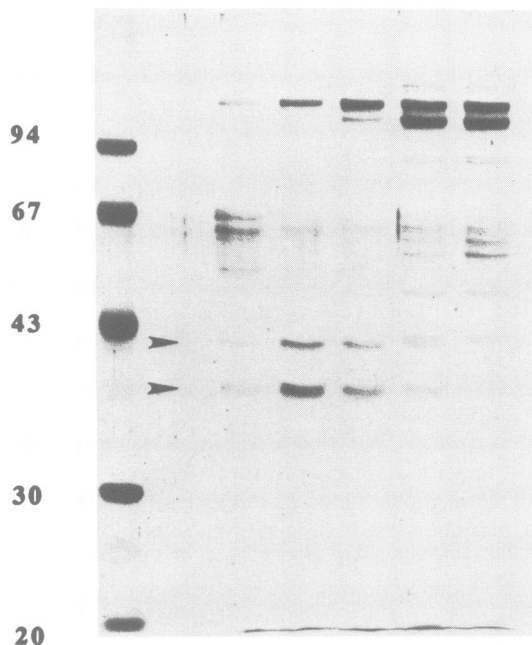
Fig. 4. Side by side analysis by SDS—polyacrylamide gel electrophoresis and Coomassie blue staining of successive fractions across the Mono Q peak of CAK activity. The left lane shows migration of molecular weight markers. Note that the 37 and 40 kDa polypeptides (arrowheads) peak with CAK activity, in contrast to a 110 kDa contaminating polypeptide.

Starfish CAK is not specific for a given type of cyclin. In the above studies, we used human cyclin A to form chimeric complexes with cdc2 or cdc2-like proteins that were substrates for starfish CAK. This was because *Escherichia coli* readily expresses this cyclin in a soluble and correctly folded form that is easy to purify with good recovery from bacterial extracts (Lorca *et al.*, 1992b). Yet, CAK was also found to phosphorylate and activate complexes between bacterially produced human cyclin B1 and either *Xenopus* cdc2 or *Xenopus* cdk2 (Figure 12, upper panel). Moreover, we did not detect segregation of any kinase that would preferentially phosphorylate and activate cdc2 in association with any one of either cyclins A or B at any step of starfish CAK purification (not shown).

We were not able to produce any cyclin other than A and B in correctly folded form in *E.coli*. However, when *Xenopus* cyclin E (M.Philippe, unpublished results) was translated in the reticulocyte lysate, it was found to elicit a small but significant activation of cdc2 and cdk2 by purified CAK (Figure 12, lower panel). No activation was detected without CAK addition. Extent of H1 kinase activation was limited, presumably because, in contrast to bacterially produced cyclins A and B, cyclin E was produced only in small amounts (<0.1 ng/μl) in the *in vitro* translation system. Indeed when a similar amount of human cyclin B1, also translated in the reticulocyte assay, was used instead of bacterially produced human cyclin B1, the extent of H1 kinase activation was very similar. In constrast *Drosophila* cyclin C (Leopold and O'Farrell, 1991) did not at all support CAK-dependent activation of either cdc2 or cdk2, although it was more efficiently translated than cyclin E in reticulocyte lysate (~1 ng/μl).
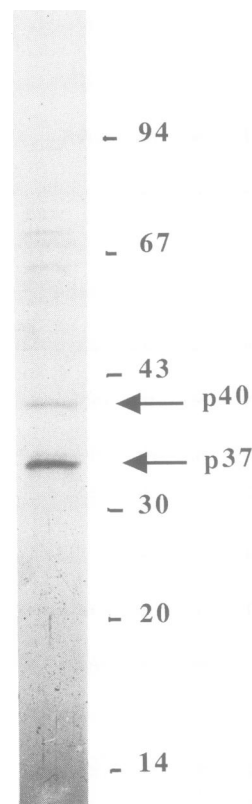
Fig. 5. Analysis by SDS—polyacrylamide gel electrophoresis and silver staining of proteins in the final peak fraction of starfish CAK. Note that unlike the routine fractionation procedure described in Figure 3, a single fraction (not the pooled peak) was loaded onto the next chromatography column during the three last steps of the purification. This reduced the yield of the preparation, but improved its purity.

Is CAK-dependent phosphorylation of cdc2/cdk2 required to confer kinase activity? Whilst no increase of H1 kinase activity was detected when cyclin A was added to cdc2 after its immunoprecipitation from *Xenopus* egg extracts, unless CAK was also added (Figure 13a), a small but significant increase of H1 kinase activity was detected when cyclin A was added to cdk2 immunoprecipitated from the same extracts, even without CAK addition. A dramatic further increase of H1 kinase activity was observed when CAK was added. ^{32}P was readily incorporated (Figure 13b) into a residue assigned to Thr160, homologue of Thr161 in *Xenopus* cdk2, when incubation with CAK was run in the presence of [γ-^{32}P]ATP (data not shown). Since no incorporation of ^{32}P into cdk2 was detected in the absence of CAK (Figure 13b), it appears that in contrast to cdc2, cdk2 may undergo partial activation upon binding cyclin A, even in the absence of Thr160 phosphorylation. Failure of cdc2 to undergo detectable activation prior to CAK addition does not appear to be due to its inability to bind cyclin A in the absence of T161 phosphorylation, since free cyclin A was removed from immunoprecipitates prior to CAK addition.

Discussion

In the present work, we purified from starfish oocytes a kinase that activates cdc2 in a cyclin-dependent manner

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XENOPUS   MLAIDCGKGAAGRMEGIAARGVDVRSRAKQYEKLDFLGEGQFATVYKARDKNTDRIVAIKKIKLGHRAEANDGINRTALREIKLLQELSHPNIIGLLDAF   100
                      ::||||||||||||
STARFISH              IEFLGEGQFATVYK
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101   GHKSNISLVFDFMETDLEVIIKDTSLVLTPAHIKSYMLMTLQGLEYLHHLWILHRDLKPNNLLLDENGVLKLADFGLAKSFGSPNRIYTHQVVTRWYRSP   200
                      ::||||||| :||||||:|||||||
                      IGDFGLAKFYGSPNRVYTHQVVTR
                      <------><-----><------->
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201   ELLFGARMYGVGVDMWAVGCIILAELLLRVPFLPGDSDLDQLTRIFETLGTPTEEQWPGMSSLPDYVAFKSFPGTPLHLIFIAAGDDLLELLQGLFTFNPC   300
                      ||||||:||||||||||:|||| |||||||||: ||||::|
                      VPFLPGESDLDQLTRIFQTLGTVTEEQWPGMTLLPDYIEF
                      <-------------><--------------------->
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301   ARCTASQALRKRYFSNRPAPTPGNLLPRPNCSIEALKEQQNLNLGIKRKRTEGMDQKDIAKKLSF   365
                      ||||||:
                      YQASQALK
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Fig. 6. Partial sequencing of the p37 component of starfish CAK as compared with the amino acid sequence of *Xenopus* MO15 (EMBL accession number X53962). The amino acids that are identical in both sequences are indicated by a vertical bar and conservative changes by two dots. Contiguous peptides are underlined.
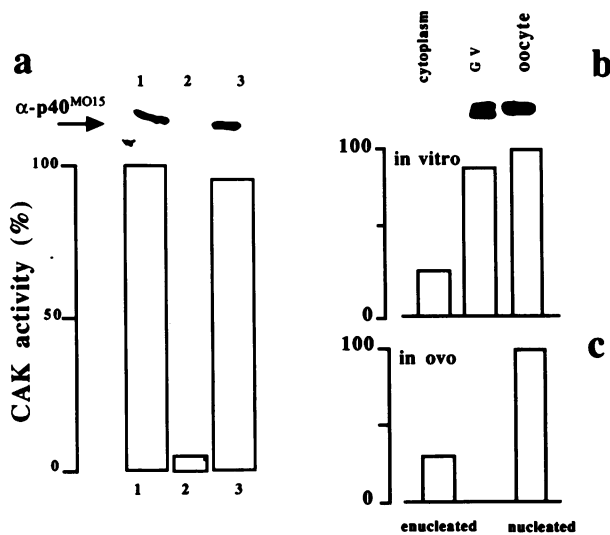

Fig. 7. The MO15 protein (p40^MO15) is associated with CAK activity in interphase extracts prepared from activated *Xenopus* eggs, as well as in prophase-arrested *Xenopus* oocytes. (a) CAK activity was assayed in an interphase extract before (1), and after immunodepletion of p40^MO15 with corresponding specific antibodies as well as in the immunoprecipitated material (3). 100% corresponds to CAK activity in the non-depleted extract. The amount of p40^MO15 protein in aliquots of extract before or after immunodepletion, as well as in the corresponding immunoprecipitate, was also estimated by Western blotting (upper panel). (b) A single oocyte was manually enucleated and the contents of either the isolated germinal vesicle (GV) or the remaining cytoplasm were homogenized in 5 μl of buffer and assayed for CAK activity *in vitro* or by Western blotting for the presence of p40^MO15. As a control (100% CAK activity), a nucleated oocyte was also assayed (the upwards shift of p40^MO15 occurs during mixing of nuclear sap with cytoplasm at homogenization). (c) Five enucleated and five nucleated *Xenopus* oocytes were microinjected with 20 nl (20 ng) each of human cyclin A. After 5 min they were homogenized in RIPA buffer, immunoprecipitated with anti-cyclin A antibodies and immunoprecipitates assayed for H1 kinase activity (the H1 kinase activity of nucleated oocytes is referred to as 100 arbitrary units).

Fig. 8. Sizing p40^MO15-containing complexes in interphase *Xenopus* extracts. An interphase *Xenopus* extract (clarified by centrifugation at 200 000 *g* for 15 min in a TL 100 Beckman centrifuge) was fractionated by gel filtration on a calibrated Superdex 200 column. Successive fractions were assayed by Western blotting for the presence of p40^MO15 (lower panel) and after 10-fold dilution for CAK activity (histogram, arbitrary units). (The 10-fold dilution lowers endogenous H1 kinase activity to undetectable level in our assay conditions.)
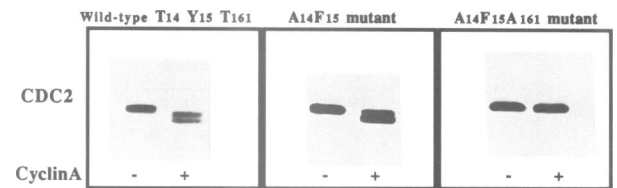
Fig. 9. Substitution of Thr161 with a non-phosphorylable residue (Ala161) abolishes phosphorylation of cdc2 by purified starfish CAK. Wild type *Xenopus* cdc2 the $A_{14}F_{15}$ double mutant and the $A_{14}F_{15}A_{161}$ triple mutant all translated from the corresponding mRNA in [35S]methionine-containing reticulocyte lysates, were treated with purified starfish CAK and ATP-Mg^{2+} in the presence (+) or absence (−) of cyclin A. ^{35}S-labelled cdc2s were immunoprecipitated after 15 min incubation, solubilized and run on 12.5% polyacrylamide gels containing SDS and submitted to fluorography. A characteristic downwards shift is observed with wild type cdc2 and the $A_{14}F_{15}$ mutant, but not the $A_{14}F_{15}A_{161}$ triple mutant.

through phosphorylation of its Thr161 residue. This phosphorylation was observed only in the presence of a cyclin, thus we believe that the target for the kinase is the cyclin−cdc2 heterodimer, not the cdc2 monomer. Since cdc2 was produced in reticulocyte lysate, however, it cannot be excluded that some unidentified factor present in the lysate could have prevented phosphorylation of cdc2 to occur in the absence of a cyclin subunit in our experimental conditions. Our most highly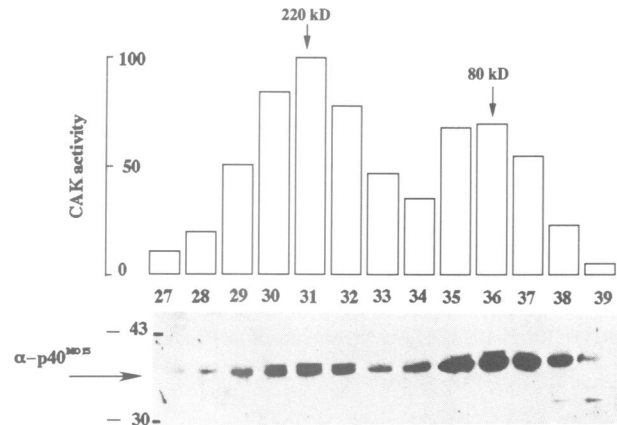 purified preparation contained only two major polypeptides, p37 and p40, which could not be separated from each other without loss of activity. Therefore the kinase appears to have been purified to a state near electrophoretic homogeneity. The purified kinase was
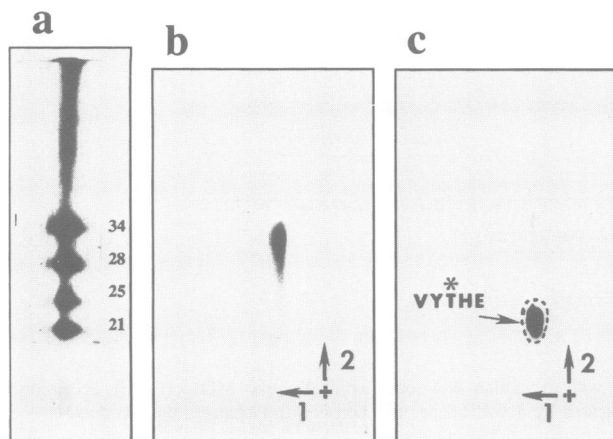
Fig. 10. Starfish CAK phosphorylates cdc2 on Thr161. (**a**) Analysis of the degradation products of cdc2 by *N*-chlorosuccinimide. cdc2, produced in reticulocyte lysate, was phosphorylated *in vitro* by purified CAK in the presence of cyclin A and [γ-^{32}P]ATP. After immunoprecipitation, cdc2 was purified by SDS−PAGE and submitted to autoradiography: fragments of 28, 25 and 21 kDa were produced. (**b**) Same experiment as in panel a, but purified cdc2 was proteolysed with trypsin. The tryptic fragments were loaded at the position indicated by the cross and separated by bidimensional analysis. First dimension (1) TLE and second dimension (2) TLC: a single labelled fragment was obtained. (**c**) Same as in panel b but the ^{32}P-labelled tryptic fragment was further digested with V8 protease. Again, a single labelled fragment was produced. The dashed circle around the radiolabelled spot indicates the position of the synthetic phosphopeptide Val-Tyr-Thr(P)-His-Glu, which corresponds to fragment 159−163 of cdc2.
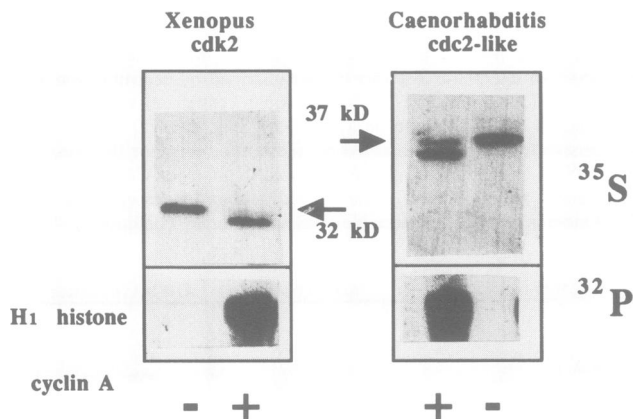

Fig. 11. Starfish CAK phosphorylates and activates *Xenopus* cdk2 as well as *Caenorhabditis* cdc2-like protein in a cyclin-dependent manner. *Xenopus* cdk2 and a *Caenorhabditis* cdc2-like protein encoded by the *ncc-1* gene were translated in the presence or absence of [^{35}S]methionine from the corresponding mRNAs in the reticulocyte lysate system. Purified starfish CAK was added, in the presence (+) or absence (−) of bacterially produced human cyclin A (0.1 μg/μl). After 15 min incubation at 25°C, cdk2 and the cdc2-like proteins were immunoprecipitated with specific antibodies. ^{35}S-labelled immunoprecipitates were solubilized in Laemmli buffer and electrophoretic mobility of the cdk2/cdc2-like proteins monitored by polyacrylamide gel electrophoresis and fluorography: a characteristic downshift of both *Xenopus* cdk2 and *Caenorhabditis* cdc2-like proteins is observed in the presence of cyclin A only. A mixture containing H1 histones and [γ-^{32}P]ATP was added to the unlabelled immunoprecipitates. After 10 min at 25°C, proteins were solubilized in Laemmli buffer and phosphorylation of H1 histones monitored by autoradiography after gel electrophoresis.
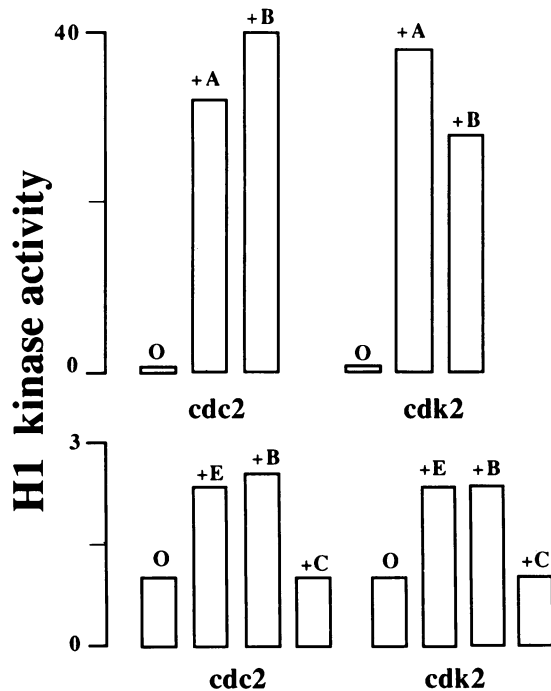

Fig. 12. Starfish CAK activates cdc2−cdk2 in chimeric complexes containing cyclins A, B or E. Cdc2 or cdk2 was immunoprecipitated from an interphase *Xenopus* extract and aliquots assayed (see Materials and methods) for activation of H1 kinase activity by purified starfish CAK in the presence of bacterially produced human cyclins A or B1 (upper panel) or reticulocyte-translated *Xenopus* cyclin E, human cyclin B1 and *Drosophila* cyclin C (lower panel). In all cases, basal H1 kinase (O, endogenous H1 kinase of immunoprecipitates plus CAK in the absence of cyclin addition) was taken as unit. Concentration of cyclins in assay was ∼50 ng/μl for bacterially produced human cyclins A and B, 0.05 ng/μl for *in vitro* translated *Xenopus* cyclin E and human cyclin B, and 0.5 ng/μl for *in vitro* translated *Drosophila* cyclin C.

found to phosphorylate and activate not only cdc2, but also cdk2 and a divergent cdc2-like protein which is encoded by the *ncc-1* gene of *Caenorhabditis elegans*, in chimeric complexes including a variety of cyclins. These include cyclin B, a component of MPF that controls mitosis, cyclin A that in association with cdk2 or cdc2 is involved in the control of both DNA replication and mitosis (Girard *et al.*, 1991; Devault *et al.*, 1992; Pagano *et al.*, 1992; Zindy *et al.*, 1992) and perhaps also in the feedback mechanism that couples mitosis to completion of DNA replication (Walker and Maller, 1991) and cyclin E, which may control the equivalent of start at late G$_1$ in higher eukaryotes (Koff *et al.*, 1991; Lew *et al.*, 1991; Dùlic *et al.*, 1992). Therefore, we propose to change the meaning of CAK from its original definition of 'cdc2-activating kinase' to 'cdk-activating kinase'. We note that cyclin C, whose companion has not yet been demonstrated to be a member of the cdc2 family, was not found to support cyclin-dependent activation of either cdc2 or cdk2 by starfish CAK. Phosphorylation by CAK was found to dramatically enhance catalytic activity of cyclin A−cdk2 complexes and to be absolutely required for expression of any H1 kinase activity of cdc2 in association with any cyclin.

We have microsequenced tryptic peptides released from either p40 or p37. Although as much as ∼1/3 of the p40 sequence was analysed, no convincing homology was found
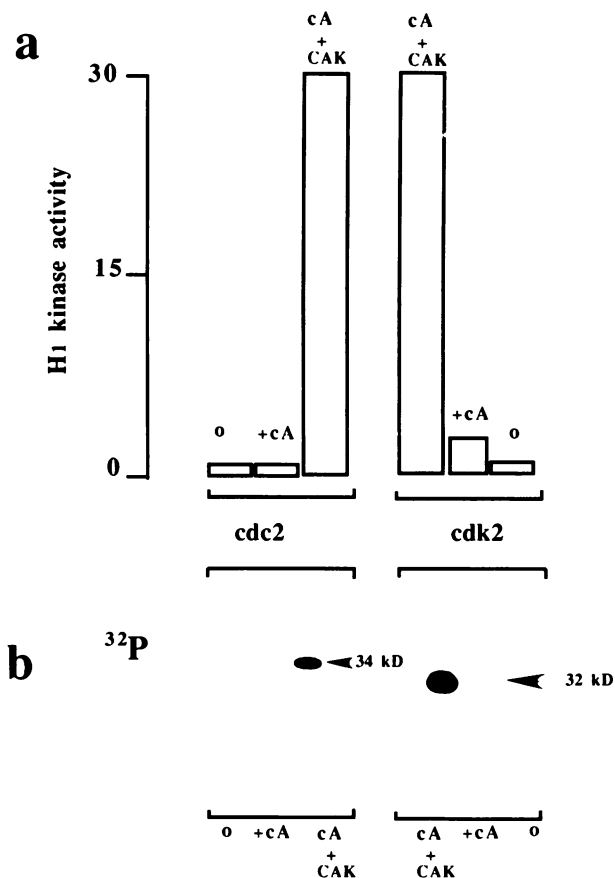
Fig. 13. CAK is absolutely required for cyclin A-dependent activation of cdc2, and dramatically increases cyclin A-dependent activation of cdk2. Upper panel, cdc2 or cdk2 were immunoprecipitated from interphase *Xenopus* extracts. Human cyclin A alone or both cyclin A and purified starfish CAK, were added to aliquots of immunoprecipitated materials in the presence of ATP-Mg^{2+}. After 15 min incubation at 25°C, immunoprecipitated materials were washed free of cyclin A and CAK, and assayed for H1 kinase activity. H1 kinase activity of untreated immunoprecipitates is taken as unit. Lower panel, [γ-^{32}P]-ATP (10 μCi) was added to 25 μl of reticulocyte lysate programmed with either *Xenopus* cdc2 or *Xenopus* cdk2, to which either cyclin A alone, cyclin A + CAK or no further material (0) were added. After 15 min incubation at 25°C, cdc2 or cdk2 were specifically immunoprecipitated and assayed for incorporation of ^{32}P by gel electrophoresis and autoradiography.

with any known protein in available data bases. In contrast, p37 is the starfish homologue of the *MO15* gene product. As expected, immunodepletion of the MO15 protein also depleted *Xenopus* egg extracts from CAK activity, which was recovered in immunoprecipitates. Taken together, the above results demonstrate that *MO15* is a gene conserved throughout evolution (at least from echinoderms to vertebrates) that encodes the catalytic subunit of the protein kinase that activates cdc2−cdks complexes through phosphorylation of Thr161 (or its homologues).

Investigations in many laboratories have demonstrated that phosphorylation of cdc2 on Thr161/167, at least in cyclin A/B-containing complexes, is essential for expression of its catalytic activity in eukaryotes, including yeast. It is therefore surprising that no mutation seems to have been as yet characterized that could identify any gene encoding CAK in yeast or *Drosophila*. Possibly, expression of a *cdc* phenotype would require mutations in several genes encoding

redundant CAKs, although we failed to detect more that one catalytic subunit in oocytes. Alternatively, the gene encoding CAK could control a distinct and as yet unidentified essential function, perhaps making mutations in this gene lethal. As a matter of fact, available data in *Xenopus* oocytes actually suggest that *MO15* may be a pleiotropic gene. Indeed, specific inhibition of P40^{MO15} synthesis in stage VI *Xenopus* oocytes was found to facilitate release from arrest in prophase of meiosis I, due to hormonal stimulation by progesterone. Since inhibition of p40^{MO15} synthesis had no such effect when oocytes were released from meiotic arrest by MPF microinjection, this led to propose that p40^{MO15} could be involved in negatively regulating meiosis (Shuttleworth *et al.*, 1990), which is obviously unexpected for a cdc2-activating kinase. It would be premature to propose any interpretation to this apparent and provocative paradox.

Dephosphorylation of Thr161 in cdc2 has been correlated with exit from mitosis (Lorca *et al.*, 1992a) and at least in DU249 chicken cells, its rephosphorylation observed at S phase (Krek and Nigg, 1991). This raises the possibility that activities that control the level of Thr161 phosphorylation might be regulated during the cycle. Although MO15 has a companion subunit and undergoes post-translational modifications by phosphorylation that appear to depend on its cytological localization in both *Xenopus* and starfish oocytes (unpublished results), nothing is known at the present time on regulation of CAK activity. Although we did not see as yet dramatic changes of CAK activity along meiotic maturation and early embryogenesis, we are concerned that nuclear localization of MO15 makes evaluation of its actual activity in whole homogenates irrelevant, in particular because of changes in its state of phosphorylation that occur when the nuclear envelope is broken down during the homogenization procedure, as observed in the present work. The cell cycle has been shown to function in early embryos derived from enucleated oocytes (Dabauvalle *et al.*, 1988; Picard *et al.*, 1988). Since CAK should be required for cdc2-kinase activation, the MO15 protein is probably actively synthesized during meiotic maturation and early embryogenesis. A more detailed understanding of how the MO15 protein works in the living cell will hopefully emerge with continuous studies on its synthesis, steady state level, cytological localization, post-translational modifications, as well as on the identification of its companion(s), which may specify its function(s). Possibly, future investigations will reveal that phosphorylation of Thr161 and its homologues in cdc2−CDKs complexes is regulated in a sophisticated manner, as previously reported for phosphorylation of MAP kinase(s) on homologous residues of the conserved domain VIII of serine-threonine kinases (reviewed by Pelech and Sanghera, 1992).

Materials and methods

Oocytes and homogenates

Prophase-arrested oocytes of the starfish *Marthasterias glacialis* were prepared free of follicle cells and of the jelly coat which surrounds the oocytes as previously described by Labbé *et al.* (1991). Oocytes were washed quickly with an ice-cold buffer containing 144 mM β-glycerophosphate, 34 mM EGTA, 27 mM MgCl2, 1.8 mM dithiothreitol (DTT), 200 mM sucrose and 200 mM KCl at pH 7.8, then pelleted by low speed centrifugation (< 100 *g* for 1 min). After adding 2 vol of the same but 2-fold diluted buffer, oocytes were crushed in a Potter homogenizer and centrifuged at 12 000 *g* for 15 min. The supernatant was collected and centrifuged again for 40 min

at 140 000 *g*. The last supernatant was frozen in liquid nitrogen and kept at −70°C until use.

Prophase-arrested *Xenopus* oocytes were isolated from an ovary fragment and prepared free of follicle cells by manual dissection with fine forceps. Enucleation procedure and procedures used to recover intact germinal vesicles and healthy enucleated oocytes have been described elsewhere by Dabauvalle *et al.* (1988). Unfertilized eggs were obtained from PMSG-primed females induced to spawn by HCG injection. Interphase extracts were prepared from dejellied eggs electrically activated in the presence of 100 μg/ml cycloheximide. 40 min after activation, eggs were washed in an excess of buffer containing 100 mM potassium acetate, 2.5 mM magnesium acetate, 7 mM EGTA, 1 mM DTT and 10 μg/ml cytochalasin B at pH 7.3 (Felix *et al.*, 1990). Excess buffer was removed and eggs crushed at 10 000 *g* for 1 h. The yellow soluble layer between the pellet and the fat cake was collected, frozen in liquid nitrogen and kept at −70°C until use.

Purification of starfish CAK
The starfish extract (300 ml) supplemented by a cocktail of proteases inhibitors (leupeptin, pepstatin, chymostatin and aprotinin, 1 mg/l of each and 1 mM PSMF) was dialysed for 4 h against 6 l of buffer A (50 mM Tris−HCl pH 7.3, 1 mM EGTA, 1 mM DTT, 1 mM sodium fluoride and 1 mM sodium orthovanadate). The dialysed extract was centrifuged at 10 000 *g* for 45 min and the resultant topmost lipid layer discarded. The clear supernatant was filtered through 5 μm, then 0.45 μm filters and was then applied onto a 100 ml SP-Sepharose fast flow column (Pharmacia) equilibrated with buffer A. Bound proteins were eluted at a flow rate of 100 ml/h by a 0−1 M linear gradient of NaCl in buffer A. Fractions of 10 ml were collected and assayed for CAK activity (see below for description of the assay). Active fractions 36−42 (see Figure 2a) were pooled and loaded onto a 10 ml heparin−Sepharose column (Pharmacia) equilibrated with buffer A. The retained proteins were eluted at 180 ml/h with a 0−1 M gradient of NaCl in buffer A. Fractions of 3 ml were collected and assayed for CAK activity. Positive fractions 35−43 (see Figure 2b) were pooled and proteins precipitated by 45% ammonium sulphate. The precipitate was collected by centrifugation and solubilized in 3 ml of buffer A. After clarification by spinning in a TL 100 Beckmann centrifuge (150 000 *g* for 15 min) it was applied on a Superdex 200 HR 16/60 gel filtration column (Pharmacia) equilibrated in buffer A containing 60 mM NaCl. Proteins were eluted (flow rate 60 ml/h) and 2 ml fractions collected. Fractions 30−32 (see Figure 2c) were pooled, diluted 4-fold in buffer A and loaded on a HR 5/5 Mono Q column (Pharmacia) equilibrated with buffer A. The retained proteins were fractionated by a 0−0.4 M linear gradient of NaCl in buffer A (flow rate: 0.8 ml/min). Fractions of 0.4 ml were collected and assayed for CAK activity. Positive fractions 31−33 (see Figure 2d) were passed through a PD10 column (Pharmacia) equilibrated with buffer B (50 mM MES pH 6.5, 1 mM EGTA and 1 mM DTT) and applied onto a HR 5/5 Mono S column (Pharmacia) equilibrated with buffer B. The retained proteins were further fractionated at a flow rate of 0.8 ml/min by a 0−0.4 M linear gradient of NaCl in buffer B. Fractions of 0.4 ml were collected and their CAK activity determined. Positive fractions 38−41 were pooled and stored as 20 μl aliquots at −80°C until use.

Expression of proteins in bacteria and the reticulocyte lysate translation system
Production in *E.coli* of human cyclin A and its purification from bacterial extracts has been described previously by Lorca *et al.* (1992b). Human cyclin B1 was expressed in *E.coli* as a fusion protein with maltose-binding protein using the pMal-c2 vector of Biolab (Beverley, USA) and purified, first on amylose resin according to manufacturer's instructions, then by ion exchange on a Mono Q column followed by gel filtration on a Superdex 200 column (both from Pharmacia). Stock solutions of bacterially produced and purified cyclins A and B1 were ~1 mg/ml.

Xenopus cdc2, cdk2 and cyclin E, the *Caenorhabditis* cdc2-like protein encoded by the *ncc-1* gene, human cyclin B1 and *Drosophila* cyclin C were transcribed by T7 or T3 (*Caenorhabditis*) polymerases and translated in the presence or absence of [³⁵S]methionine in reticulocyte lysate (Promega) programmed with the corresponding synthetic mRNAs. The $T_{14}A$, $Y_{15}F$ and $T_{14}A$, $Y_{15}F$, T161A mutants were prepared from wild-type *Xenopus* cdc2 according to Solomon *et al.* (1992). All these proteins were produced very efficiently in reticloctye lysate (1−2 ng/μl) with the exception of cyclin E, translated with an ~10-fold lesser efficiency.

CAK and H1 histone kinase activities
Fractions to be assayed for CAK activity (2 μl) were added to 1 μl of reticulocyte lysate containing *in vitro* translated *Xenopus* cdc2 (~1 ng/μl), 1 μl of a mixture containing 1 mM ATP and 50 mM MgCl2, and either 1 μl water (control) or 1 μl (0.1 μg) of bacterially produced and purified human cyclin A (or other cyclins as specified in text). After 15 min at 25°C,

samples were diluted by adding water (25 μl) and immediately assayed for H1 kinase activity by adding 10 μl of a reaction mixture containing 1 mg/ml H1 histones, 40 mM HEPES pH 7.4, 40 mM MgCl₂ and 1 mM [γ-³²P]ATP (100 c.p.m./pmol). The reaction was allowed to proceed for 10 min at 25°C and arrested by spotting on a disk of P81 phosphocellulose paper that was extensively washed, dried, and counted in a liquid scintillation counter. In some experiments, immunoprecipitates from *Xenopus* interphase extract were used instead of *in vitro* translated cdc2 as a source of cdc2−cdk substrate. In this case immunoprecipitates were first resuspended in 1 vol of a mixture containing purified CAK, 1 mM ATP, 20 mM MgCl₂ and the specified cyclin (or no cyclin, control). After 15 min at 25°C, immunoprecipitates were recovered by low speed centrifugation, washed several times with 50 mM Tris pH 7.4−150 mM NaCl, and finally assayed as above for H1 kinase activity.

Antibodies, Western blots and immunoprecipitations
Production of the polyclonal antiserum directed against *Xenopus* p40^*MO15* has been described previously (Shuttleworth *et al.*, 1990). Antibodies against *Xenopus* cdc2 and cdk2 and against the *Caenorhabditis* cdc2-like protein encoded by the *ncc-1* gene were produced by immunizing rabbits with synthetic peptides corresponding to the respective C-terminal amino acids sequences of these proteins (C8, *Xenopus*; C18, *Caenorhabditis*).

For Western blot analysis, we used the E.C.L. chemiluminescent method (Amersham, UK) according to the manufacturer's instructions. Immunoprecipitations were performed after diluting extracts in RIPA buffer (150 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 10 mM sodium phosphate, 10 mM β-glycerophosphate, 0.05% sodium deoxycholate, 1% Nonidet NP-40 and 1 mM DTT at pH 7.4) and immune complexes were isolated on protein A−Sepharose. They were resuspended and washed several times, first with RIPA buffer, then with 50 mM Tris pH 7.4 before use.

Phosphoamino acids analysis and peptide mapping
Labelling of *Xenopus* cdc2−cdk2 was preformed by adding [γ-³²P]ATP (10 μCi) to the reaction mixture containing purified CAK and cyclin A used to phosphorylate immunoprecipitated cdc2−cdk2. After 15 min at 25°C, immunoprecipitates were recovered by low speed centrifugation, washed several times with RIPA buffer then 50 mM Tris pH 7.4 and solubilized in Laemmli buffer. Proteins were separated by gel electrophoresis in the presence of SDS and either transferred to an Immobilon membrane (phosphoamino acids analysis) or directly submitted to autoradiography in order to localize labelled cdc2 before excision (peptide mapping). Procedures used for phosphoamino acids analysis, chemical and proteolytic cleavages, as well as for peptide mapping have been described previously by Lorca *et al.* (1992a).

Amino acid sequencing
Polypeptides were electroblotted onto nitrocellulose (Schleicher and Schuell) and digested *in situ* by bovine pancreatic TPCK-trypsin (4 μg) overnight at 37°C (Aebersold *et al.*, 1987). The released peptide mixture was injected onto an Aquapore C₈ reverse-phase microbore column (0.21 × 10 cm; Brownlee) equilibrated in 0.1% TFA and was eluted with a 0−60% acetonitrile gradient. Peaks were further purified by reverse-phase HPLC using the same conditions but a C₁₈ column. Peptides from reverse phase HPLC were loaded onto a Polybrene-coated glass fibre disc and inserted into an Applied Biosystems model 470 gas phase microsequencer coupled on line with a model 120 A PTH analyser. The standard 03R PTH program was used.

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