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p40^{MO15} associates with a p36 subunit and requires both nuclear translocation and Thr176 phosphorylation to generate cdk-activating kinase activity in *Xenopus* oocytes

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p40^{MO15}, a cdc2-related protein, is the catalytic subunit of the kinase (CAK, cdk-activating kinase) responsible for Thr161/Thr160 phosphorylation and activation of cdk1/cdk2. We have found that strong overexpression of p40^{MO15} only moderately increases CAK activity in *Xenopus* oocytes, indicating that a regulatory CAK subunit (possibly a cyclin-like protein) limits the ability to generate CAK activity in p40^{MO15} overexpressing oocytes. This 36 kDa subunit was microsequenced after extensive purification of CAK activity. Production of *Xenopus* CAK activity was strongly reduced in enucleated oocytes overexpressing p40^{MO15} and p40^{MO15} shown to contain a nuclear localization signal required for nuclear translocation and generation of CAK activity. p40^{MO15} was found to be phosphorylated on Ser170 and Thr176 by proteolytic degradation, radiosequencing of tryptic peptides and mutagenesis. Thr176 phosphorylation is required and Ser170 phosphorylation is dispensable for p40^{MO15} to generate CAK activity upon association with the 36 kDa regulatory subunit. Finally, Thr176 and Ser170 phosphorylations are not intramolecular autophosphorylation reactions. Taken together, the above results identify protein–protein interactions, nuclear translocation and phosphorylation (by an unidentified kinase) as features of p40^{MO15} that are required for the generation of active CAK.

Key words: CAK/cell cycle/cyclin-dependent kinase/nuclear translocation/*Xenopus* oocyte

Introduction

Transitions of the cell cycle are controlled in all eukaryotes by sequential formation, activation and subsequent inactivation of kinase complexes between cyclin-dependent kinases (cdks) belonging to the p34$^{cdc2/CDC28}$ family of proteins, and regulatory cyclin subunits (for reviews, see Hunt, 1991; Norbury and Nurse, 1992; Pines, 1993; Sherr, 1993; Dorée and Galas, 1994).

The activity of cdks is regulated along the cell cycle not only by association with cyclins and a variety of recently discovered low molecular weight inhibitors (reviewed by Hunter, 1993), but also by phosphorylation/dephosphorylation reactions. To date, only phosphorylation sites of cdk1 (p34$^{cdc2/CDC28}$), cdk2 and more recently cdk4 (Kato *et al.*, 1994) have been identified. They include Tyr15 and Thr14 whose phosphorylation, which is regulated by the balanced activities of the wee1$^+$/mik1$^+$ protein kinases, an unidentified membrane-associated protein kinase (Kornbluth *et al.*, 1994) and members of the cdc25 family of dual specificity phosphatases (reviewed by Gautier, 1993), is inhibitory for cdk1 and cdk2.

By contrast, phosphorylation of Thr161 (human cdk1) and homolog Thr residues in cdk2 and cdk4 appears to be required for cdk1, cdk2 and cdk4 activation (reviewed by Draetta, 1993; Pines, 1993), even though a low but significant activity can be detected *in vitro* in unphosphorylated cdk2–cyclin A (Connell-Crowley *et al.*, 1993) and cdk1–cyclin A complexes (M.Dorée, unpublished result). The kinase responsible for Thr161/Thr160 phosphorylation of cdk1/cdk2, first partially purified from *Xenopus* eggs (Solomon *et al.*, 1992), has been further purified from this material (Solomon *et al.*, 1993) and to apparent homogeneity from starfish oocytes (Fesquet *et al.*, 1993). The kinase, called CAK (for cdk-activating kinase) has been shown to phosphorylate cdk1 or cdk2 associated with cyclin A, B or E (Fesquet *et al.*, 1993; Poon *et al.*, 1993). Whether it phosphorylates cdk4 is still unclear (Kato *et al.*, 1994). CAK comprises two proteins: the first, the homolog of the *MO15* gene product, is a conserved p40 protein kinase related to the p34$^{cdc2/CDC28}$ family of proteins (Shuttleworth *et al.*, 1990 ; Darbon *et al.*, 1994), the second protein is believed to be a regulatory subunit although its partial sequencing in starfish did not reveal any convincing homology with any known protein (Fesquet *et al.*, 1993).

In the present work, we have investigated how CAK activity is generated after p40^{MO15} synthesis in *Xenopus* oocytes. We find that generation of CAK activity from newly synthesized p40^{MO15} requires its nuclear translocation, phosphorylation of a unique residue identified as Thr176 and association with a partially microsequenced p36 regulatory subunit.

Results

Strong overexpression of p40^{MO15} only moderately increases CAK activity in Xenopus oocytes

mRNAs *in vitro* transcribed from a *Xenopus MO15* cDNA-containing plasmid optimized for efficient translation (see Materials and methods), were microinjected into stage VI oocytes incubated in the presence of 250 μCi/ml [^{35}S]-methionine. At various times after microinjection, individual oocytes were crushed and the resulting homogenates analyzed after SDS–PAGE for expression of p40^{MO15} by fluorography (Figure 1a) or immunoblot, using a polyclonal antibody (C-ter antibody) directed against its

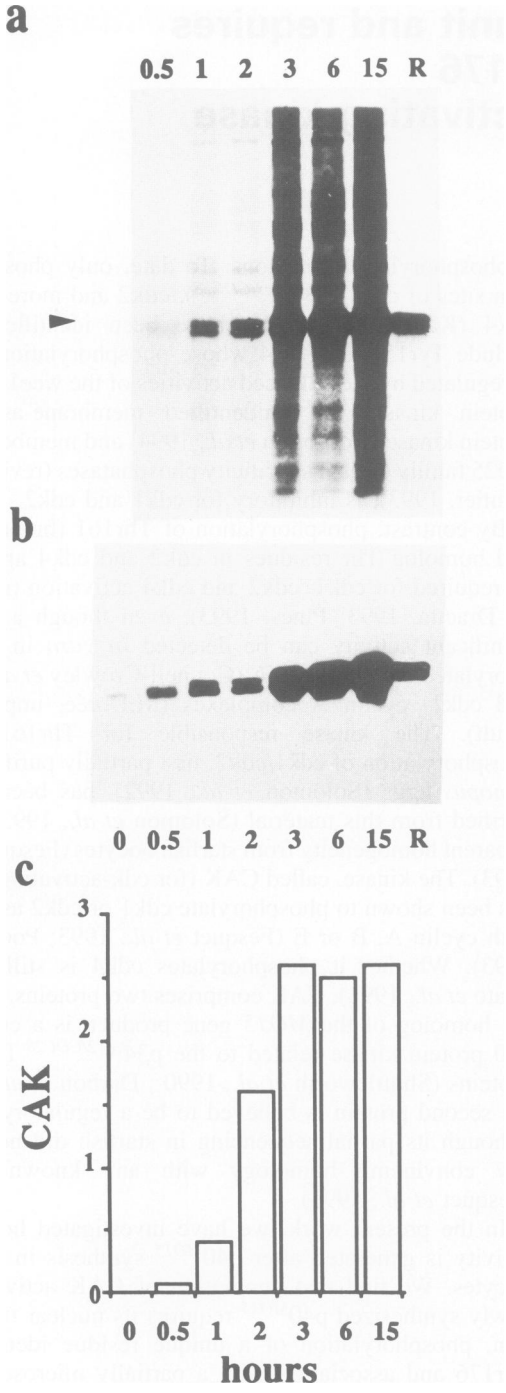

Fig. 1. Strong overexpression of p40^{MO15} only moderately increases CAK activity in *Xenopus* oocytes. *MO15* mRNA was microinjected into stage VI *Xenopus* oocytes incubated in the presence of [^{35}S]methionine. At the indicated times (h) after microinjection, oocytes were crushed and the resulting homogenates analyzed for expression of p40^{MO15} after SDS−PAGE and for potential increase of CAK activity due to p40^{MO15} overexpression. (**a**) Fluorogram showing incorporation of [^{35}S]methionine into p40^{MO15} (arrowhead). R, the *MO15* mRNA was translated in reticulocyte lysate and the translation product run on the same gel. (**b**) The same experiment as in (a), but expression of p40^{MO15} was monitored by immunoblotting with a polyclonal antibody (C-ter) directed against its C-terminus. The level of endogenous p40^{MO15} is shown on the left (zero time). (**c**) CAK activities generated by overexpression of p40^{MO15} are plotted against times after mRNA microinjection. The level of CAK activity in control, non-overexpressing oocytes is taken as unit (thus 1 on the *y*-axis corresponds to a 2-fold increase of CAK activity as compared with control oocytes).

C-terminal 15 amino acid sequence (Figure 1b). The full-length *Xenopus MO15* mRNA contains two potential start sites coding for predicted proteins of 41 and 40 kDa respectively and analysis of proteins synthesized by oocytes from the endogenous mRNA indicates that the second AUG is in fact used preferentially *in vivo* (Shuttleworth *et al.*, 1990). Microinjected mRNAs were translated extremely efficiently in oocytes but, at variance with the endogenous mRNA, the first AUG appeared to be used as efficiently as the second one. As soon as 30 min after microinjection the amount of ectopically translated p40^{MO15} was already 3- to 8-fold higher than that of the endogenous protein and it further accumulated later on. After overnight incubation (~15 h) it exceeded by 100- to 150-fold the amount of endogenous p40^{MO15}.

Triplicate aliquots were taken from the same homogenates to monitor potential increase of CAK activity due to p40^{MO15} overexpression. They were used to activate complexes formed *in vitro* between recombinant human GST-cdk2 and cyclin A in standard conditions. Then complexes were diluted and assayed for determination of generated H1 kinase activity, from which CAK activity of each homogenate was deduced. In the standard conditions used (see Materials and methods) it was checked that CAK activity, as determined using the above assay, reproducibly reflected the extent of threonine phosphorylation of cdk2, as determined by SDS−PAGE and autoradiography (Fesquet *et al.*, 1993). As shown in Figure 1c, overexpression of p40^{MO15} was found in this experiment to generate an additional CAK activity as high as 2.5-fold that of control oocytes (either non-injected or sham-injected). Yet CAK activation was rather limited as compared with the extent of p40^{MO15} overexpression. Moreover, maximal CAK activity was reached between 2 and 3 h after microinjection, whilst p40^{MO15} continued to accumulate thereafter. The above experiment was repeated using 12 different females. All of them readily translated microinjected mRNAs, resulting in a level of ectopically produced p40^{MO15} at least 50-fold higher than that of the endogenous protein after overnight incubation. In four of them, no significant change of CAK activity was detected as compared with the control. In five other females, a significant increase of CAK activity was clearly detected, although it did not exceed a 2-fold ratio as compared with control oocytes. Finally, in three females CAK activity was increased by as much as 2- to 4-fold as compared with control oocytes. In these experiments the extent of CAK activation in oocytes overexpressing p40^{MO15} did not appear to be correlated with the period of the year when females were used, whether primed with PMSG (pregnant mare serum gonadostimulin) or not. Similar variations in the extent of CAK activation were observed upon addition of p40^{MO15}, *in vitro* translated in reticulocyte lysate, to oocyte extracts prepared from different females (data not shown). We also constructed a p40^{MO15} mutant that initiates only at the second ATG. No obvious difference in generation of CAK activity was observed as compared with the wild type when this mutant was expressed in oocytes, although only the lower band of the p40^{MO15} doublet accumulated in microinjected oocytes (data not shown).

The above results show first, that the p40^{MO15} protein, as translated *in ovo* from microinjected mRNAs, is correctly

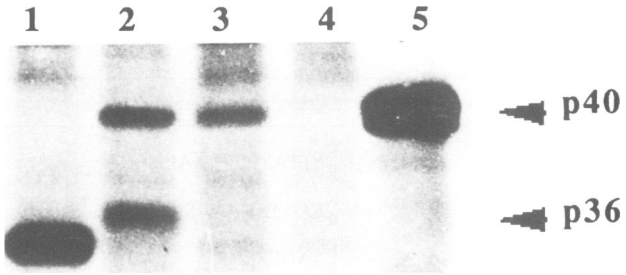

Fig. 2. Association of p36 with $p40^{MO15}$ in active CAK immunoprecipitates. Homogenates prepared from [^{35}S]methionine-labeled oocytes were immunoprecipitated in the absence of SDS (lane 2) with a polyclonal antibody (C-ter) directed against the C-terminus of *Xenopus* $p40^{MO15}$ and the immunoprecipitated material analyzed by SDS−PAGE and fluorography. Arrowheads point to the *MO15* protein (p40) and the associated p36 component. Lane 3, same experiment, but the homogenate was first treated for 30 min with 0.1% SDS before immunoprecipitation; lane 4, same experiment as in lane 2 but the antibody was first saturated with the antigene peptide encoding the C-terminal sequence of $p40^{MO15}$; lane 1 (control), the same homogenate was immunoprecipitated with a polyclonal antibody raised against the C-terminus of *Xenopus* $p34^{cdc2}$; lane 5, [^{35}S]methionine-labeled $p40^{MO15}$, translated in reticulocyte lysate from the corresponding mRNA, was run on the same gel.

folded and can generate CAK activity and second, that another factor, possibly a regulatory CAK subunit, limits the ability of ectopically produced $p40^{MO15}$ to generate CAK activity in overexpressing oocytes. In all overexpression experiments thereafter, females generating CAK activity upon ectopic expression were used exclusively.

$p40^{MO15}$ is associated with a 36 kDa polypeptide in active CAK

We have previously purified CAK from starfish oocytes to near homogeneity and shown that the starfish homolog of $p40^{MO15}$ is associated with a 41 kDa protein from which it could not be separated without loss of activity (Fesquet *et al.*, 1993).

In the next experiment, maturing *Xenopus* oocytes were incubated for 8 h with 500 μCi/ml [^{35}S]methionine and homogenates immunoprecipitated, in the absence of SDS, with the C-ter antibody. As shown in Figure 2 (lane 2), two proteins (p40 and p36) were immunoprecipitated and specifically disappeared when the antibody was first saturated with a peptide encoding the C-terminal sequence of $p40^{MO15}$ (compare lanes 2 and 4). The 36 kDa protein was not a slowly migrating form of *Xenopus* cdk1, since it was not immunoprecipitated by antibodies directed against the C-terminus of this protein (lane 1). When homogenates were treated with 0.1% SDS before immunoprecipitation, only $p40^{MO15}$ was recovered in immunoprecipitates (lane 3). We conclude that p36 interacts with $p40^{MO15}$ and is not directly recognized by the $p40^{MO15}$C-ter antibody. Immunoprecipitates comprising p36 and $p40^{MO15}$ were found to readily activate H1 kinase activity of cdk1, cdk2 and cdk3 in chimeric complexes with cyclin A and activation of each of these complexes was associated with the paradoxical increase of mobility that characterizes phosphorylation of Thr161 in cdk1 or equivalent residues in other cdks (shown for cdk3 in Figure 3a and b).

The above results suggested that p36 might be the regulatory subunit, equivalent to the 41 kDa starfish protein, that seems to limit CAK activity generated by

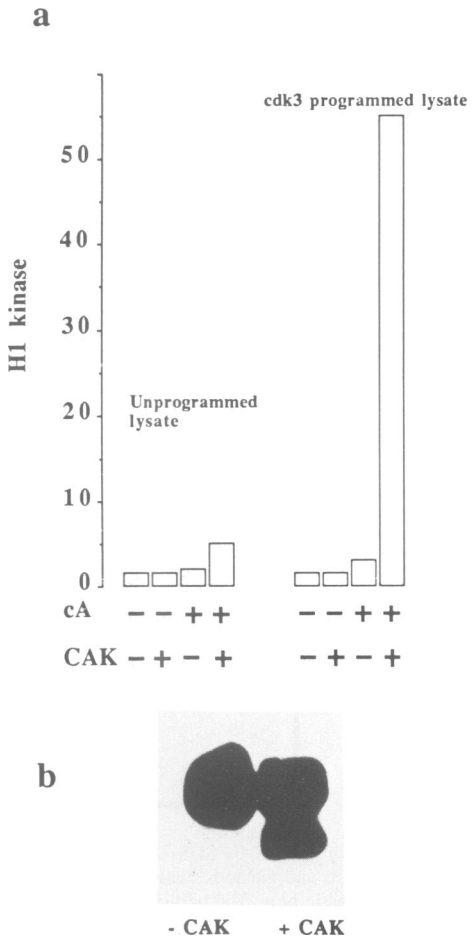

Fig. 3. Immunoprecipitated p36−$p40^{MO15}$ complexes activate H1 kinase activity of cdk3 in chimeric complex with cyclin A. Cdk3 was translated in reticulocyte lysate from the corresponding mRNA in the presence of [^{35}S]methionine and phosphorylated by immunoprecipitated CAK in the presence of cyclin A. (**a**) H1 histone kinase activity of cdk3−cyclin A complex (arbitrary units) after incubation with immunoprecipitated CAK. Reticulocyte lysate, programmed or not with cdk3 mRNA, was assayed for H1 kinase activity after incubation (10 min, 25°C) in the presence (+) or absence (−) of recombinant human cyclin A (cA, 0.1 mg/ml) and/or either immunoprecipitated CAK (+CAK) or protein A−Sepharose (−CAK). (**b**) Phosphorylation of cdk3 by immunoprecipitated CAK increases its electrophoretic mobility. Same experiment as in a, but *in vitro* translated cdk3 was analyzed by SDS−PAGE and fluorography after (+CAK) or before (−CAK) its phosphorylation by immunoprecipitated CAK in the presence of cyclin A.

ectopically overexpressing $p40^{MO15}$ in *Xenopus* oocytes. To get more information about p36 identify, we purified CAK from *Xenopus* eggs, using a simplified procedure derived from methods previously used to purify starfish CAK (see Materials and methods). Briefly, CAK was first partially purified by conventional column chromatography on Q Fast flow-Sepharose and heparin-Fast flow-Sepharose, and further purified by immunoaffinity on a matrix of C-ter affinity-purified antibodies cross-linked to protein A−Sepharose. Finally, purified CAK, eluted with 0.1% trifluoro-acetic acid was analyzed by SDS−PAGE (Figure 4). Only two major proteins were detected. One was $p40^{MO15}$, the other was the p36 associated subunit that was digested *in situ* with trypsin after electroblotting. The released peptides were separated and four of them microsequenced (Table I). No significant homology was

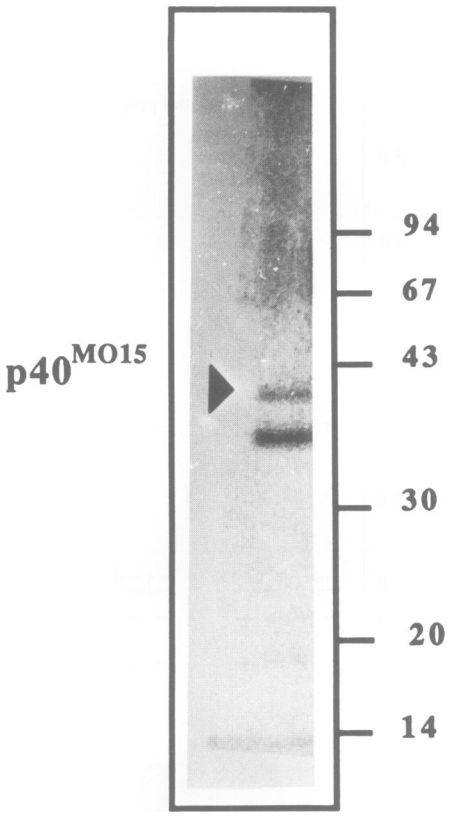

Fig. 4. Analysis of *Xenopus* CAK by SDS–PAGE. The purified preparation (see Materials and methods) was analyzed by SDS–PAGE and stained with Coomassie blue. The p40 component (arrowhead) was recognized, after electroblotting, by the polyclonal antibody raised against $p40^{MO15}$. The p36 component was digested with trypsin and partially microsequenced (see Table I).

found between the microsequenced peptides and any known protein in available databases.

Overexpressed $p40^{MO15}$ requires nuclear translocation to generate CAK activity

We previously presented evidence that both endogenous $p40^{MO15}$ and CAK activities are associated with the large nucleus known as 'germinal vesicle' in stage VI *Xenopus* oocytes (Fesquet *et al.*, 1993).

In the next experiment, we examined the localization of ectopic $p40^{MO15}$ and the generated CAK activity in oocytes incubated in the presence of [^{35}S]methionine. Oocytes were enucleated at various times after cytoplasmic microinjection of mRNAs and both $p40^{MO15}$ and the generated CAK activity quantified in either the cytoplasmic or the nuclear compartment. As shown in Figure 5a, the $p40^{MO15}$ doublet first appeared in cytoplasm and began to accumulate significantly in the nucleus after 1 h. Three hours after mRNAs microinjection, nearly equivalent amounts of ectopic $p40^{MO15}$ were found to have accumulated in cytoplasm and nucleus respectively. Although nuclear translocation of $p40^{MO15}$ still occurred thereafter, about one half of ectopic $p40^{MO15}$ was still found in cytoplasm 8 h after microinjection, perhaps due to saturation of the nuclear translocation system in the presence of sustained *MO15* mRNA translation. Indeed when cytoplasm was taken at that time and transferred into the cytoplasm of non-mRNA microinjected oocytes, most ectopic $p40^{MO15}$ had moved to the germinal vesicle of recipient oocytes within 1 h (not shown). The amount of translocated $p40^{MO15}$ by far exceeds that of endogenous p36, thus nuclear translocation of $p40^{MO15}$ certainly does not require its association with the p36 subunit.

Table I. Partial microsequencing of the p36 component of *Xenopus* CAK

Microsequenced peak[a]	Determined sequence
a	K/R YEPAYPEEVAALK
b	K/R KGYEDDGYISK
c	K/R SADEFLNR
d	K/R VDEFNVSSIQFVGNLGENPL

[a]Peptides resulting from trypsin degradation were separated by HPLC on a reverse phase microbore column before microsequencing.

In the above experiments, most ectopically generated CAK activity was found within the germinal vesicle at any one time (Figure 5b), as was endogenous CAK activity. This may indicate that the p36 subunit is itself a nuclear protein and that $p40^{MO15}$ must first enter the nucleus for CAK activity to be generated. In the next experiment, mRNAs were microinjected into enucleated oocytes. Although such oocytes readily accumulated $p40^{MO15}$, they generated poor CAK activity (Figure 6). Possibly this low but significant activity originates from association of ectopic $p40^{MO15}$ with newly synthesized endogenous p36 in enucleated oocytes.

Xenopus $p40^{MO15}$, as well as the recently cloned goldfish and human homologs (Onoe *et al.*, 1993; Darbon, *et al.*, 1994) contains a potential nuclear localization signal (NLS) at the C-terminal end, i.e. the KRKR motif (Lys334–Arg337 in *Xenopus* $p40^{MO15}$). Besides a cluster of at least three out of five basic residues, bipartite nuclear localization signals also contain a doublet of two basic residues 10 amino acids upstream of this cluster (Robbins *et al.*, 1991). Interestingly, a conserved KK doublet separated by a variable 10 amino acid spacer (nine in goldfish) from the KRKR motif was observed in $p40^{MO15}$, although downstream of the basic cluster (Figure 7). To assess the role of both the KRKR cluster and the KK doublet in nuclear translocation of ectopically produced $p40^{MO15}$ and its ability to generate CAK activity, we first mutated the KK doublet to NN, or deleted the C-terminus of the protein by creating a stop codon just upstream from the sequence encoding the KRKR motif (see Materials and methods). None of these constructs were useful. Indeed, mutation of the KK doublet did not prevent nuclear translocation of $p40^{MO15}$ and the C-terminally deleted protein, although readily translated in reticulocyte lysate, failed to accumulate in *Xenopus* oocytes (data not shown). We then mutated the KRKR motif to QALR. As shown in Figure 8, this mutation completely suppressed both nuclear translocation of $p40^{MO15}$ and generation of CAK activity in stage VI oocytes.

$p40^{MO15}$ is phosphorylated on Thr176 and Ser170 in active CAK complexes

In preliminary experiments *Xenopus* oocytes, either arrested at stage VI or induced to mature *in vitro* with

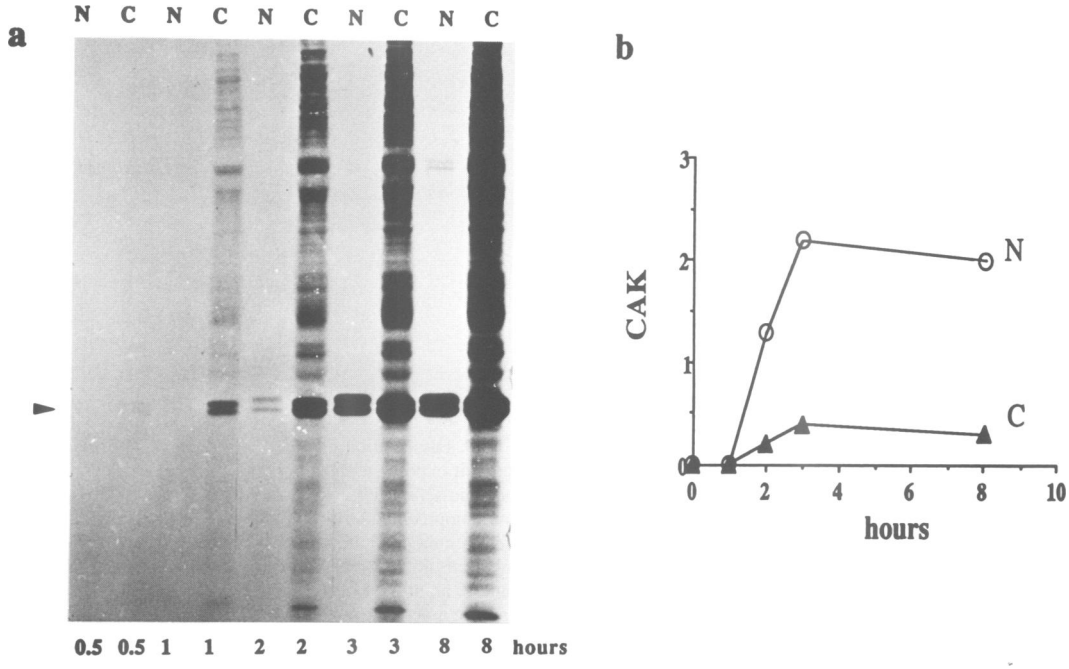

Fig. 5. Localization of ectopic p40^{MO15} and the generated CAK activity in *Xenopus* oocytes microinjected with *in vitro* transcribed *MO15* mRNA. (**a**) Oocytes were enucleated at the indicated times after microinjection and p40^{MO15} accumulation (arrowhead) in either the cytoplasmic (C) or the nuclear (N) compartments monitored by SDS−PAGE and fluorography. (**b**) CAK activities generated by overexpression of p40^{MO15} were quantified in the same nuclear (N) or cytoplasmic (C) homogenates. The level of CAK activity in whole, non-overexpressing oocytes is taken as unit. The slight different position of the p40^{MO15} doublet, depending on whether it is nuclear or cytoplasmic, is believed to be due to the considerable difference in the amount of proteins loaded in the corresponding lanes (~0.2 μg per nuclear as compared with 15 μg per cytoplasmic lane).

progesterone, were incubated overnight in the presence of ^{32}P, p40^{MO15} was then immunoprecipitated from homogenates with the C-ter antibody and analyzed for incorporation of ^{32}P by SDS−PAGE and autoradiography. In these experiments no significant incorporation of ^{32}P into p40^{MO15} was detected, except in one experiment where a slight but significant phosphorylation was observed on serine residue(s) only (data not shown). In the course of these early experiments, we made the intriguing observation that electrophoretic mobility of p40^{MO15} strikingly depends on the way samples are prepared; when oocyte extracts are first treated with 1% SDS (or Laemmli sample buffer) even for only 30 s at room temperature before boiling, mobility of p40^{MO15} is reduced (Figure 9, lane 2). The shift does not occur either when boiling Laemmli buffer is directly added (lane 3), when extracts are first treated with 1 μM okadaic acid before detergent addition (lane 4), or when p40^{MO15} immunoprecipitates (washed free of their supernatants) are treated directly with Laemmli buffer (lane 1). Our interpretation of these results is that dissociation of p40^{MO15} by detergent from an associated subunit (presumably p36) exposes a phosphorylated site to an okadaic-sensitive phosphatase (resistant to 1% SDS).

Dephosphorylation of this site reduces p40^{MO15} mobility, as paradoxically observed for dephosphorylation of Thr161 in cdk1 and Thr160 in cdk2 (Lorca *et al.*, 1992a; Rosenblatt *et al.*, 1992). Moreover, *Xenopus* p40^{MO15} contains a threonine residue (Thr176, starting translation from the second AUG) at the equivalent position of Thr161 in cdk1, cdk2 and cdk3 (Figure 10). This suggested that Thr176 of p40^{MO15} might be phosphorylated in the active p40^{MO15}−p36 complex although the phosphorylated residue, not accessible to the relevant phosphatase in the

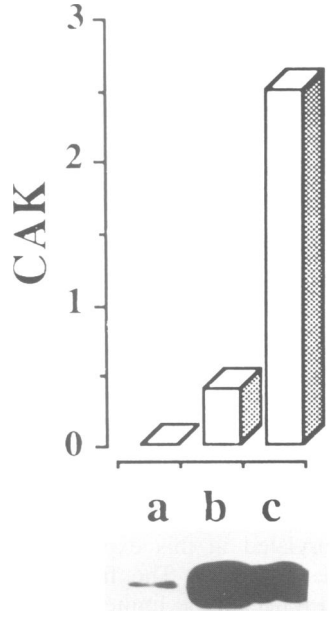

Fig. 6. Enucleated oocytes poorly generate CAK activity, although they can readily overexpress p40^{MO15}. Histogram: CAK activity generated during the 8 h period after *MO15* mRNA microinjection in enucleated (b) and whole oocytes (c). (a) non-microinjected nucleated oocytes. The level of CAK activity in control, non-overexpressing oocytes is taken as unit. CAK activities were estimated from 20 oocytes, either nucleated or enucleated. The immunoblot below the histograms shows the extent of p40^{MO15} accumulation in the same oocytes.

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                334                 348
Xe MO15  LNLGIKRKRTEGMDQKDIAKKLSF
Hu MO15  PALAIKRKRTEALEQGGLPKKLIF
GF MO15  LLIGIKRKR.DSIEQGTLKKKLVF
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Fig. 7. Alignment of C-terminal ends of *Xenopus* (Xe), human (Hu) and goldfish (GF) *MO15*. The NLS and the doublet of basic residues are boxed.

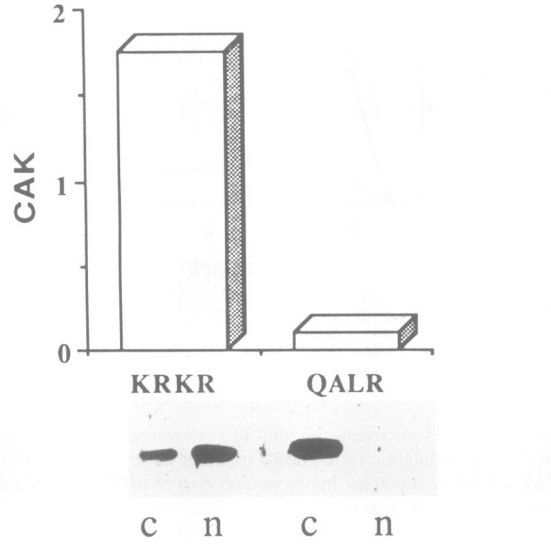

Fig. 8. Overexpression of a NLS mutant (KRKR→QALR) fails to generate CAK activity. The wild type (KRKR) or the mutant (QALR) *MO15* mRNAs were microinjected into the cytoplasm of stage VI oocytes. After 12 h homogenates were prepared from 25 oocytes of each category and CAK activities generated by expression of microinjected mRNAs measured, taking as unit CAK activity of non-microinjected oocytes. Accumulation after 12 h expression of the wild type and mutant proteins in the cytoplasm (c) or the nucleus (n) of injected oocytes is shown in the lower panel.

CAK complex, did not turn over and could not be labelled *in ovo*.

A prediction of the above interpretation was that p40^{MO15} should, in contrast, be readily phosphorylated on Thr176 if oocytes were incubated with ^{32}P at the time when new CAK complexes were assembled. In the next experiment, stage VI oocytes were first microinjected with *MO15* mRNA, then incubated in the presence of 1 mCi/ml [^{32}P]orthophosphate, or 500 μCi/ml [^{35}S]methionine. After 8 h, p40^{MO15} was immunoprecipitated with the C-ter antibody and analyzed by SDS−PAGE and autoradiography. As shown in Figure 11a (lane 4) p40^{MO15} was indeed phosphorylated in this experiment and the p36 subunit to a lesser extent. The limited extent of p36 phosphorylation reflected the limited amount of the non-overexpressed protein in immunoprecipitates, as shown by [^{35}S]methionine labeling (lane 3). Phosphoamino acid analysis revealed the presence of ^{32}P-labeled phosphoserine and, to a lesser extent, of ^{32}P-labeled phosphothreonine in p40^{MO15} (Figure 11b). Only ^{32}P-labeled phosphoserine was detected in p36 (not shown). When immunoprecipitated p40^{MO15} was extensively degraded by trypsin, only two ^{32}P-labeled fragments were produced, one contained only phosphoserine, the other one only phosphothreonine (Figure 11c). Finally, when immunoprecipitated p40^{MO15} was treated with the V8 protease and

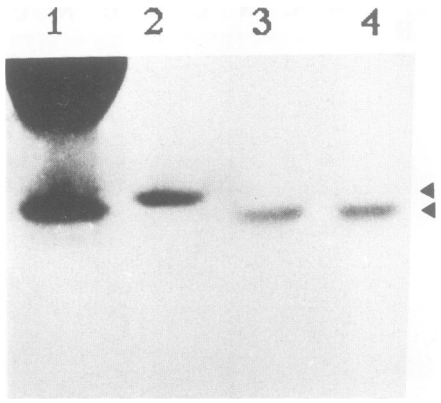

Fig. 9. Dissociation of p40^{MO15} by detergent from an associated subunit exposes a phosphorylated site, most likely Thr176, to an okadaic-sensitive phosphatase. Lane 1, p40^{MO15} was immunoprecipitated (in the absence of SDS) from oocyte extract and the immunoprecipitated material analyzed by SDS−PAGE and Western blotting with the C-ter antibody against *Xenopus* p40^{MO15}; lane 2, an aliquot of the same oocyte extract was treated for 30 s at room temperature with 0.5% SDS before boiling and analyzed by SDS−PAGE and Western blotting with the same antibody; lane 3, boiling Laemmli buffer was added directly to another aliquot of the same oocyte extract, which was also analyzed by SDS−PAGE and Western blotting; lane 4, same experiment as in lane 2, but 1 μM okadaic acid was added before SDS. Arrowheads point to p40^{MO15}.

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                 162      170      176
MO15 - XL  DFGLAKSFGSPNRIYTHQVVTRWYRSPE
cdk1-XL    DFGLARAFGIPVRVYTHEVVTLWYRAPE
cdk2-XL    DFGLARAFGVPVRTFTHEVVTLWYRAPE
cdk3-Hu    DFGLARAFGVPVRTFTHEVVTLWYRAPE
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Fig. 10. Alignment of the V8 proteolytic fragments spanning Thr176 in *Xenopus MO15* (*MO15-XL*) and homologous residues in *Xenopus* cdc2 (cdk1-XL), *Xenopus* cdk2 (cdk2-XL) and human cdk3 (cdk3-Hu). The V8 protease and trypsin cut *Xenopus MO15* at positions indicated by bars and arrowheads, respectively. Phosphorylated residues are shown in bold letters.

the resulting peptide fragments analyzed by HPLC on a C8 Aquapore column, a single peak of radioactive material was recovered (not shown).

There is a single V8 proteolytic fragment (peptide 162–188, see Figure 10) in the *Xenopus* p40^{MO15} sequence spanning a serine and a threonine residue that can be separated by trypsin digestion. It includes Ser170 and Thr176. Although the 162–188 peptide also includes Ser167 as a potential phosphoacceptor residues, Ser170 was the best candidate because its phosphorylation had been detected previously when highly purified starfish CAK was partially microsequenced (Fesquet *et al.*, 1993). Identification of Thr176 and Ser170 as *in vivo* phosphorylated residues was further confirmed by radiosequencing the two ^{32}P-labeled tryptic peptides generated from p40^{MO15}. As expected, radioactivity was eluted at the third and the fourth rounds of radiosequencing respectively. No attempt was made to estimate the number of phosphorylated serine residues in the p36 CAK subunit.

Thr176 phosphorylation is required and Ser170 phosphorylation dispensable for p40^{MO15} to generate CAK activity

To investigate the role of Thr176 and Ser170 phosphorylation in generating CAK activity from overexpressed

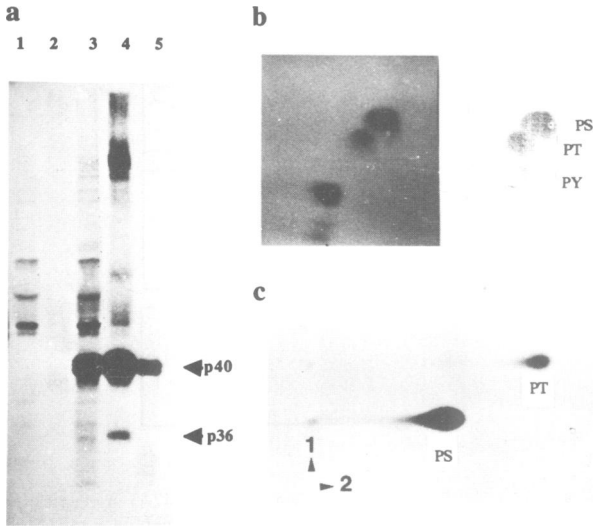

Fig. 11. p40^{MO15} is phosphorylated on a serine and a threonine residues localized on two different tryptic fragments. Oocytes microinjected with *MO15* mRNA were incubated in the presence of either [^{32}P]orthophosphate or [^{35}S]methionine. After 8 h, p40^{MO15} was immunoprecipitated with the C-ter antibody and analyzed by SDS−PAGE and autoradiography. (**a**) Homogenates, prepared from ^{35}S-labeled oocytes (1 and 3) or ^{32}P-labeled oocytes (2 and 4) were immunoprecipitated with immune serum directed against p40^{MO15} (3 and 4) or the pre-immune serum (1 and 2). Lane 5, ^{35}S-labeled p40^{MO15}, *in vitro* translated in reticulocyte lysate, was run on the same gel. Arrowheads point to p40^{MO15} and the associated p36 component. Note that besides p36, most ^{35}S-labeled polypeptides associated with p40^{MO15} in immunoprecipitates are also recovered in pre-immunoprecipitates and thus correspond to contaminating material. (**b**) Immunoprecipitated ^{32}P-labeled p40^{MO15} was electroblotted on Immobilon after SDS−PAGE and processed for determination of phosphoamino acids after acid hydrolysis. Phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY) were separated by bi-dimensional thin layer electrophoresis at pH 1.9 in the first and pH 3.5 in the second dimension. Position of non-labeled marker phosphoamino acids (included in the labeled sample), after staining with ninhydrin, is shown on the right; position of ^{32}P-labeled amino acids generated by acid hydrolysis is shown after autoradiography on the left. (**c**) ^{32}P-labeled immunoprecipitated p40^{MO15} was electroblotted on nitrocellulose after SDS−PAGE and extensively degraded by trypsin. The resulting material was analyzed by thin layer electrophoresis in the first and TLC in the second direction followed by autoradiography. ^{32}P-labeled amino acids of each tryptic spot were determined as above after acid hydrolysis and bidirectional analysis.

p40^{MO15}, these sites were mutated to non-phosphorylatable alanine residues and the corresponding mRNAs microinjected into stage VI oocytes. As shown in Figure 12a, the Thr176→Ala 176 mutation abolished production of CAK activity and, as expected, suppressed labeling of phosphothreonine in ectopic p40^{MO15} (Figure 12b). In contrast production of CAK activity was not reduced, and even slightly increased in the Ser170→Ala 170 mutant, as compared with wild type p40^{MO15} (Figure 12a), although serine phosphorylation was suppressed in the mutant (Figure 12c). Thus, although both Thr176 and Ser170 are phosphorylated in active CAK, only phosphorylation of Thr176 is required for CAK activity. The finding that Ser170 phosphorylation is dispensable and perhaps even slightly detrimental for CAK activity is somewhat surprising. We did not investigate further the role of Ser170 phosphorylation.

As noted above, Thr176 is localized at the equivalent position of Thr161 in cdc2 (Thr167 in fission yeast). In fission yeast, *ts* cdc2 mutants that overexpress cdc2-Glu167 accumulate at mitosis, suggesting that Glu167 mimics constitutive phosphorylation of Thr167 and supports cdc2 kinase activation (Ducommun *et al.*, 1991; Gould *et al.*, 1991). This prompted us to investigate the effect of mutating Thr176 to Glu176 in p40^{MO15}. As shown in Figure 12a, no significant CAK activity was generated when this mutant was overexpressed in oocytes.

Although Thr176 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 highly purified starfish CAK (data not shown). This suggested that Thr176 phosphorylation may not be an autophosphorylation reaction. To confirm this view, we used a mutant in which Lys47 was changed to Ala47. This mutant still underwent phosphorylation on serine and threonine residues when overexpressed in oocytes (Figure 12d), although no significant CAK activity was generated (Figure 12a). Since Lys47 is the equivalent in p40^{MO15} of a lysine residue conserved in all protein kinases that is required for transfer of phosphate from ATP to the protein substrate (Hanks *et al.*, 1988), our interpretation of this result is that neither phosphorylation of Ser170 nor that of Thr176 result from an intramolecular autophosphorylation reaction.

Discussion

The aim of this work was to investigate how CAK activity is generated from p40^{MO15} after its translation in *Xenopus* oocytes. We found that strong overexpression of p40^{MO15} in microinjected oocytes from *in vitro* transcribed mRNA increased CAK activity only to a limited extent, suggesting that another factor, possibly a regulatory CAK subunit, limits the ability of ectopically produced p40^{MO15} to generate CAK activity in overexpressing oocytes. In agreement with this view, a p36 component was found to be specifically associated with p40^{MO15} when an antibody directed against the C-terminal tail of p40^{MO15} was used to immunoprecipitate CAK activity. Four tryptic peptides were microsequenced from p36 (56 amino acids). No convincing homology was found between the microsequenced peptides and any known protein in available databases. Using oligonucleotides derived from the microsequenced peptides, we recently isolated by PCR a partial cDNA (600 bp) potentially encoding the p36 component of *Xenopus* CAK (data not shown). Interestingly it presents some similarity with cyclin C. Work is in progress to obtain the complete clone. We previously purified CAK from starfish oocytes to near homogeneity and showed that the starfish homolog of p40^{MO15} is associated with a 41 kDa protein (Fesquet *et al.*, 1993). Although seven different peptides derived from p41 were sequenced, including a total of 136 amino acids, no significant homology was found with any known protein. We recently cloned starfish p41 and obtained its complete sequence (Devault *et al.*, manuscript in preparation). *Xenopus* p36 does not have any homology with starfish p41. Thus we do not know at the present time if the same regulatory subunit(s) associate(s) with the *MO15* protein in active CAK complexes purified from starfish and *Xenopus* oocytes.

Xenopus p40^{MO15} contains at its C-terminal end a

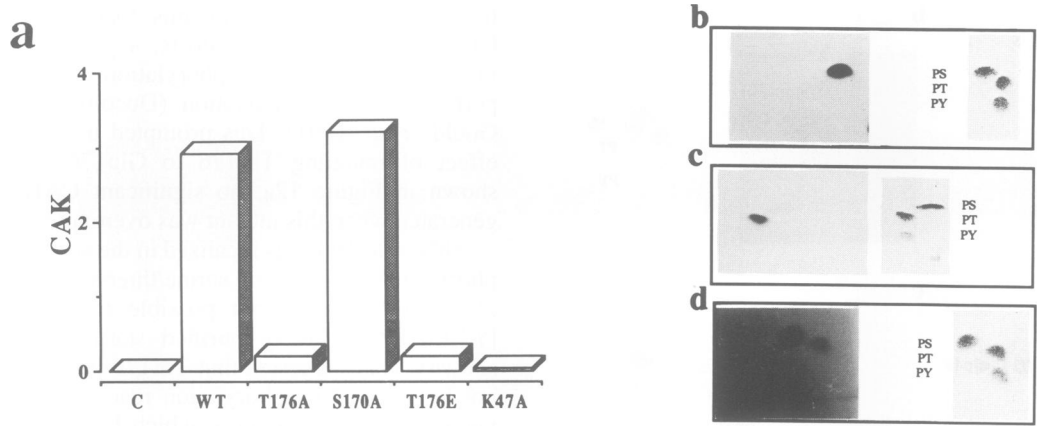

Fig. 12. Production of CAK activity following overexpression of wild type (WT) and mutant p40^{MO15} (T176A, S170A, T176E, K47A) in oocytes microinjected with the corresponding mRNAs. (**a**) Twelve hours after mRNA microinjection, groups of 30 oocytes microinjected with each mRNA were homogenized (see Materials and methods) and used for determination of CAK activity generated in oocytes. The level of expression of each mutant was very similar to that of wild type p40^{MO15} in microinjected oocytes (see Figure 1). The level of CAK activity in control (c) non-overexpressing oocytes is taken as unity for generated CAK activities. (**b**), (**c**) and (**d**) in parallel with the above experiments, oocytes microinjected with the T_{176}A (b), S_{170}A (c) and K_{47}A (d) mutant mRNAs were incubated with [^{32}P]orthophosphate. After 15 h incubation, overexpressed p40^{MO15} mutants were immunoprecipitated and analyzed as above for ^{32}P-labeled amino acids (see legend to Figure 11) after SDS−PAGE, electroblotting and acid hydrolysis. Position of ^{32}P-labeled amino acids is shown after autoradiography on the left; position of non-labeled marker phosphoamino acids, by staining by ninhydrin, is shown on the right.

potential nuclear localization signal, conserved throughout evolution, i.e. the KRKR motif, 10 amino acids upstream of a KK doublet. Whilst mutation of the KK doublet to NN was not sufficient to prevent nuclear translocation of ectopically produced p40^{MO15}, mutation of the KRKR motif to QALR completely suppressed both nuclear translocation of p40^{MO15} and generation of CAK activity in oocytes. Generation of CAK activity was also dramatically reduced in enucleated oocytes overexpressing p40^{MO15}. These results strongly suggest that p40^{MO15} must first enter the nucleus for an active CAK complex to be formed. We also monitored nuclear accumulation of ectopic p40^{MO15} and localization of the generated CAK as a function of time following *MO15* mRNA microinjection. Whilst p40^{MO15} clearly accumulated in the nucleus, a significant proportion of the ectopic protein was also found at any time in the cytoplasm, either due to nucleocytoplasmic shuttling (Schmidt-Zachmann *et al.*, 1993) or saturation of the nuclear translocation system by sustained p40^{MO15} synthesis. Even under such conditions, most ectopically generated CAK activity was found within the nucleus. This may indicate that the p36 subunit is a nuclear protein and that p40^{MO15} must first enter the nucleus for an active CAK complex to be formed. Alternatively, the p40^{MO15}−p36 complex may form in cytoplasm and undergo activation after nuclear translocation (see below). According to either of these views, the low but significant amount of newly generated CAK activity detected in cytoplasm might reflect nucleocytoplasmic shuttling of the kinase complex.

The *MO15* gene product was first discovered in *Xenopus* as a member of the cdc2 family of protein kinases, although its expression failed to rescue cdc2 function in yeast mutants (Shuttleworth *et al.*, 1990). Interestingly, it contains a threonine residue (Thr176 in *Xenopus*), conserved from starfish to human, localized at the equivalent position of Thr161 in cdc2 and other cdks. Phosphorylation of Thr161 in cdc2 or equivalent residues in other cdks is required for full activation of all known cdk−cyclin complexes, perhaps because it facilitates substrate binding to the catalytic site of these protein kinases (reviewed in Pines, 1993; Draetta, 1993; Doree and Galas, 1994). This led us to investigate the possible phosphorylation of Thr176 in p40^{MO15} and its possible role in CAK activity.

The *MO15* protein was indeed found to be phosphorylated on Thr176 in immunoprecipitated CAK complexes, as shown by proteolytic degradation and radiosequencing of tryptic peptides generated from p40^{MO15}. To investigate the role of Thr176 phosphorylation in generating CAK activity, this residue was mutated to alanine and the corresponding mRNA microinjected into oocytes. The Thr176 Ala mutation suppressed generation of CAK activity in microinjected oocytes, thus phosphorylation of Thr176 is necessary for p40^{MO15} to form active CAK complexes. Thr176 phosphorylation does not appear to be an intramolecular autophosphorylation reaction since the kinase-dead Lys47 Ala mutant still underwent phosphorylation on Thr176 when overexpressed in oocytes. We do not know what kinase (nuclear? cytoplasmic?) is responsible for Thr176 phosphorylation, but future investigations may reveal that activity of CAK complexes is regulated in a sophisticated manner through Thr176 phosphorylation, as previously reported for phosphorylation of MAP kinases on homologous residues of the conserved domain VIII of serine-threonine kinases (reviewed by Mordret, 1993).

Besides Thr176, p40^{MO15} was found to be phosphorylated only on one other single residue that was mapped as Ser170. In contrast to Thr176 phosphorylation, Ser170 phosphorylation is not required for CAK activity, since oocytes overexpressing the Ser170 Ala p40^{MO15} mutant readily generated CAK activity. We previously detected phosphorylation of the homologous residue of Ser170 whilst microsequencing purified starfish CAK (Fesquet *et al.*, 1993) thus, Ser170 is certainly phosphorylated in active CAK. Yet, ^{32}P-labeling of Ser170 was systematically

found in the present work to exceed that of Thr176. This may indicate that, at variance with Thr176, Ser170 may undergo phosphorylation in monomeric $p40^{MO15}$. Alternatively, Thr176 phosphorylation might be stabilized in CAK complexes only. Although the role of Ser170 phosphorylation remains unknown, we note that this site is a potential phosphoacceptor for a member of the cdks or MAP kinase families.

Materials and methods

Oocytes, microinjections and enucleation procedure

Stage VI oocytes were isolated from an ovary fragment and prepared free of follicle cells by manual dissection with fine forceps. Aliquots (50 nl) of solution containing 1 mg/ml mRNA in water were injected into the cytoplasm of oocytes, as described by Dorée *et al.* (1993). Enucleation procedure and procedures used to recover intact germinal vesicles and healthy enucleated oocytes have been described elsewhere by Dabauvalle *et al.* (1988).

Plasmid contructs and RNA synthesis

The wild type *Xenopus MO15* cDNA, subcloned into the SP6 transcription vectors SP64R1 (Krieg and Melton, 1984), the corresponding *K47R* and *T176A* mutant as well as the plasmid encoding human cdk3 were generous gifts of John Shuttleworth, Randy Poon and Li-Huei Tsai, respectively. The $K_{348}K_{345} \rightarrow N_{348}N_{349}$ double mutant and the *ΔNLS* mutant deleted of the 17 amino acid C-terminus were constructed by a PCR method essentially as described by Herlize and Koenen (1990), with the following oligonucleotides and their sense counterparts :

KK→NN : 5′-GGAATTCAAAAACTCAAATTATTAGCA-3′
ΔNLS : 5′-GGAATTCTTTCTCTATATTCCCAG-3′

The $S_{170}A$, $T_{176}E$ and $K_{334}R_{335}K_{336} \rightarrow Q_{334}A_{335}L_{336}$ mutants were constructed by single stranded directed mutagenesis, essentially as described by Kunkel *et al.* (1987) using the following antisens oligonucleotides:

$S_{170}A$: 5′-CTGTTTGGACGCCCAAATG-3′
$T_{176}E$: 5′-CACTTGATGCTCATATATTCT-3′
KRK→QAL : 5′-CATGCCTTCTGTCCGCAAGGCCTGTATTCCCA-GGTTGAG-3′

All constructs were checked by direct sequencing. Capped *MO15* mRNAs were synthesized as described by Krieg and Melton (1987) and adjusted to 1 mg/ml after sequential precipitation with LiCl and EtOH.

CAK activities

Stage VI oocytes (or groups of oocytes) were homogenized (1 oocyte/10 µl) in 10 mM Tris pH 7.5–1 mM EGTA and centrifuged for 3 min at 10 000 *g* to remove yolk. Triplicate aliquots of 2 µl were taken from the supernatant with a precise micropipet and added to 3 µl of a mixture containing 0.33 mM ATP, 16.6 mM $MgCl_2$, 0.1 µg GST-cdk2 and 0.1 µg cyclin A (both produced in *Escherichia coli*, as described in Lorca *et al.*, 1992a). After 30 min at 25°C, samples were diluted by adding 25 µl distilled water and assayed as previously described (Labbé *et al.*, 1991) for H1 histone kinase activity by adding 10 µl of a mixture containing 4 mg/ml histone H1, 80 mM HEPES pH 7.4, 40 mM $MgCl_2$ and 1 mM [γ-^{32}P]ATP (100 c.p.m./pmol). Since stage VI oocytes only contain a low H1 histone kinase activity (Labbé *et al.*, 1988) and basal H1 histone kinase activity of non-phosphorylated GST-cdk2–cyclin A complexes is ~80-fold lower than that of their Thr160-phosphorylated counterparts (Connell-Crowley *et al.*, 1993), at least 95% of the measured H1 histone kinase was generated during the 30 min incubation of GST-cdk2–cyclin A complexes with the diluted oocyte extracts. CAK activity of oocyte extracts was deduced from the increased H1 histone kinase activity of GST-cdk2–cyclin A complexes, as determined from triplicate samples. It was checked in preliminary experiments that, in the standard conditions of our assay, increases of H1 histone kinase activities were directly proportional to the extent of Thr160 phosphorylation in the cdk2 moiety of GST-cdk2, as quantitatively determined by incorporation of ^{32}P into the fusion protein after SDS–PAGE, autoradiography and counting of radioactivity associated with the GST-cdk2 spot in a liquid scintillation counter. The above assay was used to quantify the effect of overexpressing $p40^{MO15}$ (or derived mutants) in oocytes on CAK activity. In each experiment, manually defollicularized control oocytes from the same batch (and of the same size) were microinjected with 50 nl of distilled water to allow accurate comparison with oocytes microinjected with 50 nl of each mRNA solution.

Immunoblotting and immunoprecipitation of MO15 protein

Expression of wild type and mutant *MO15* proteins was monitored by using a polyclonal antibody (C-ter antibody) raised in rabbits against a 15 amino acid peptide corresponding to the C-terminus of *Xenopus MO15*. In a few experiments, when the above antibody could not be used (*ΔNLS* mutant), we used another polyclonal antibody, raised against the last 122 amino acids of *Xenopus MO15*, that was kindly provided by John Shuttleworth (Shuttleworth *et al.*, 1990).

To immunoprecipitate the *MO15* protein from [^{35}S]methionine- or [^{32}P]orthophosphate-labeled oocytes, homogenates (100 µl) were diluted with 1.4 ml of a buffer containing 50 mM Tris pH 7, 1 mM EGTA, 300 mM NaCl and 0.5% Nonidet NP-40, and clarified by centrifugation at 10 000 *g* for 5 min before adding the C-ter antibody. Immunoprecipitates were recovered by standard methods using protein A–Sepharose. In some experiments (see text) SDS was added to the final concentration of 0.1% in homogenates to dissociate $p40^{MO15}$ from p36 prior to buffer addition and immunoprecipitation.

Phosphoamino acids analysis, peptide mapping and amino acid sequencing

^{32}P-labeled immunoprecipitates were solubilized in Laemmli buffer and proteins separated by gel electrophoresis in the presence of SDS and either transferred to an Immobilon membrane (phosphoamino acid analysis) or to a nitrocellulose membrane (peptide mapping) and submitted to autoradiography to localize $p40^{MO15}$ and/or p36. Procedures used for phosphoamino acids analysis, proteolytic cleavages, peptide mapping and amino acid sequencing have been described previously by Lorca *et al.* (1992b) and Fesquet *et al.* (1993). Radiosequencing was performed as described by Parten *et al.* (1994).

Purification of Xenopus CAK

The procedure was derived from that used previously to purify CAK from starfish oocytes (Fesquet *et al.*, 1993). Unfertilized eggs, obtained from females injected with human chorionic gonadotrophin, were dejellied by cysteine treatment and homogenized in 1 vol of 80 mM β-glycerophosphate, 15 mM EGTA, 1 mM DTT, 10 mM $MgCl_2$, at pH 7.3. The homogenate was first centrifuged at 12 000 *g* for 30 min at 4°C, The top yellow layer was discarded and the supernatant centrifuged again at 100 000 *g* for 60 min. The soluble fraction was filtered under vacuum on 5.0 µm membranes (Millipore), frozen in liquid nitrogen, and kept at −70°C until use. The homogenate (100 ml) diluted 2-fold with buffer A (50 mM Tris–HCl, 1 mM EGTA, pH 8), was loaded onto a 100 ml Q-Fast flow-Sepharose 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 (total volume 500 ml) of NaCl in buffer A. Fractions of 10 ml were collected and assayed for CAK activity. CAK activity-containing fractions were pooled and loaded onto a 10 ml heparin–Sepharose Fast flow column equilibrated in buffer A. After washing with 50 ml of buffer A, the retained proteins were eluted with 0–1 M NaCl gradient in buffer A (total volume 100 ml) at a flow rate of 180 ml/h. Fractions of 3 ml were collected and tested for CAK activity. Positive fractions were pooled and precipitated by adding 0.264 g/ml ammonium sulfate. The resulting precipitate was collected by centrifugation, solubilized in 2.5 ml of buffer A and centrifuged at 200 000 *g* for 40 min in a Beckman TL100 centrifuge. The supernatant was loaded onto a PD 10 column (Pharmacia) equilibrated in buffer B (50 mM Tris–HCl, pH. 7.5, 150 mM NaCl, 0.05% Tween 20). The first 2.5 ml were discarded and the 3.5 ml eluate was saved.

Final purification of the CAK preparation was achieved using immunoaffinity column chromatography. The affinity matrix was prepared by coupling the affinity-purified C-ter antibody directed against *Xenopus* $p40^{MO15}$ to protein A–Sepharose, as described by Schneider *et al.* (1982). The saved fraction after PD10 desalting was incubated 4 h at 4°C with the above matrix (rotation end over end). After extensive washing first with buffer B, then with water, bound proteins were eluted with 0.1% trifluoroacetic acid. The eluted material was lyophilized and its components analyzed by SDS–PAGE and electroblotting.

References

Connell-Crowley,L., Solomon,M.J., Wei,N. and Wade Harper,J. (1993) *Mol. Biol. Cell*, **4**, 79–92.

Dabauvalle,M.C., Dorée,M., Bravo,R. and Karsenti,E. (1988) *Cell*, **52**, 525–533.
Darbon,J.M., Devault,A., Taviaux,S., Fesquet,D., Martinez,A.M., Galas,S., Cavadore,J.C., Dorée,M. and Blanchard,J.M. (1994) *Oncogene*, in press.
Dorée,M. and Galas,S. (1994) *FASEB J.*, in press.
Dorée,M., Lorca,T. and Picard,A. (1993) In Fantes,P. and Brooks,R. (ed.), *The Cell Cycle. A Practical Approach.* IRL Press, Oxford, pp. 285–309.
Draetta,G. (1993) *Trends Cell Biol.*, **3**, 287–289.
Ducommun,B., Brambilla,P., Felix,M.A., Franza,B.J., Karsenti,E. and Draetta,G. (1991) *EMBO J.*, **10**, 3311–3319.
Fesquet,D., Labbé,J.C., Derancourt,J., Capony,J.P., Galas,S., Girard,F., Lorca,T., Schuttleworth,J., Dorée,M. and Cavadore,J.C. (1993) *EMBO J.*, **12**, 3111–3121.
Gautier,J. (1993) *Adv. Prot. Phosphatases*, **7**, 153–171.
Gould,K.L., Moreno,S., Owen,D.J., Sazer,S. and Nurse,P. (1991) *EMBO J.*, **10**, 3297–3309.
Hanks,S.K.,Quin,A.M. and Hunter,T. (1988) *Science*, **245**, 45–52.
Herlitze,S. and Koenen,M. (1990) *Gene*, **91**, 143–147.
Hunt,T. (1991) *Semin. Cell Biol.*, **2**, 213–222.
Hunter,T. (1993) *Cell*, **75**, 839–841.
Kato,J.Y., Matsuoka,M., Strom,D.K. and Sherr,C.J. (1994) *Mol. Cell. Biol.*, **14**, 2713–2721.
Kornbluth,S., Sebastian,B., Hunter,T. and Newport,J. (1994) *Mol. Biol. Cell*, **5**, 273–282.
Krieg,P.A. and Melton,D.A. (1984) *Nucleic Acids Res.*, **12**, 7057–7070.
Krieg,P.A. and Melton,D.A. (1987) *Methods Enzymol.*, **155**, 397-415.
Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Labbé,J.C., Picard,A., Karsenti,E. and Dorée,M. (1988) *Dev. Biol.*, **127**, 157–169.
Labbé,J.C., Cavadore,J., C. and Doree,M. (1991) *Methods Enzymol.*, **200**, 291–301.
Lorca,T. *et al.* (1992b) *J. Cell Sci.*, **102**, 55–62.
Lorca,T., Labbe,J.C., Devault,A., Fesquet,D., Capony,J.P., Cavadore,J.C., Le Bouffant,F. and Dorée,M. (1992a) *EMBO J.*, **11**, 2381–2390.
Mordret,G. (1993) *Biol. Cell*, **79**, 193–207.
Norbury,C. and Nurse,P. (1992) *Annu. Rev. Biochem.*, **61**, 441–470.
Onoe,S., Yamashita,M., Kajiura,H., Katsu,Y., Jianquao,J. and Nagahama,Y. (1993) *Biomed. Res.*, **14**, 441–444.
Parten,B.F., Mc Dowell,J.H., Nawzocki,J.P. and Hargrave,P.A. (1994) In Crabb,J.W. (ed.), *Techniques In Protein Chemistry.* Academic Press, New.York, pp. 159–167.
Pines,J. (1993) *Curr. Biol.*, **3**, 544–548.
Poon,R.Y.C., Yamashita,K., Adamczewski,J.P., Hunt,T. and Shuttleworth,J. (1993) *EMBO J.*, **12**, 3123–3132.
Robbins,J., Dilworth,S.M., Laskey,R.A. and Dingwall,C. (1991) *Cell*, **64**, 615–623.
Rosenblatt,J., Gu,Y. and Morgan,D.O. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 2824–2828.
Schmidt-Zachmann,M.S., Dargemont,C., Kühn,L.C. and Nigg,E.A. (1993) *Cell*, **74**, 493–504.
Schneider,C., Necoman,R.A., Sutherland,R., Asser,U. and Greaves,M.F. (1982) *J. Biol. Chem.*, **257**, 10766–10769.
Sherr,C.J. (1993) *Cell*, **73**, 1059–1065.
Shuttleworth,J., Godfrey,R. and Colman,A. (1990) *EMBO J.*, **9**, 3233–3240.
Solomon,M.J., Lee,T.H., and Kirschner,M.W. (1992) *Mol. Cell. Biol.*, **3**, 13–27.
Solomon,M.J., Harper,J.W. and Shuttleworth,J. (1993) *EMBO J.*, **12**, 3133–3142.

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Note added

We have now obtained a full-length cDNA encoding the p36-associated subunit; its sequence identifies the CAK regulatory subunit of *Xenopus* oocytes as a member of a new class of type C-related cyclins. While this work was in proof, the human homolog of *Xenopus* p36 (cyclin H) was identified (*Cell*, **78**, 713–724). Its identity with *Xenopus* cyclin H is 69%.