Cell cycle regulation of the p34\textsuperscript{cdc2}/p33\textsuperscript{cdk2}-activating kinase p40\textsuperscript{MO15}

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SUMMARY

A key component of Cdc2/Cdk2-activating kinase (CAK) is p40\textsuperscript{MO15}, a protein kinase subunit that phosphorylates the T161/T160 residues of p34\textsuperscript{cdc2}/p33\textsuperscript{cdk2}. The level and activity of p40\textsuperscript{MO15} were essentially constant during cleavage of fertilised Xenopus eggs and in growing mouse 3T3 cells, but serum starvation of these cells reduced both the level and activity of p40\textsuperscript{MO15}. Although the level and activity of endogenous p40\textsuperscript{MO15} did not vary in the cell cycle, we found that bacterially expressed p40\textsuperscript{MO15} was activated more rapidly by M-phase cell extracts than by interphase cell extracts. Bacterially expressed p40\textsuperscript{MO15} was phosphorylated mainly on serine 170 (a p34\textsuperscript{cdc2} phosphorylation site) by mitotic cell extracts, but mutation of S170 to alanine did not affect the activation of p40\textsuperscript{MO15}, whereas mutation of T176 (the equivalent site to T161/T160 in p34\textsuperscript{cdc2}/p33\textsuperscript{cdk2}) abolished the activation of p40\textsuperscript{MO15}. These studies suggest that the level and activity of p40\textsuperscript{MO15} is probably not a major determinant of p34\textsuperscript{cdc2}/p33\textsuperscript{cdk2} activity in the cell cycle, and that the activation of p40\textsuperscript{MO15} may require phosphorylation on T176.

Key words: CAK, cell cycle, cyclin-dependent kinase

INTRODUCTION

Cyclins and cyclin-dependent kinases (CDKs) are key regulators of the eukaryotic cell cycle (reviewed by Norbury and Nurse, 1992). In higher eukaryotes, two CDKs appear to drive M-phase and G\textsubscript{1}/S-phase in conjunction with different cyclin subunits. Cyclin B and p34\textsuperscript{cdc2} are components of maturation promoting factor (MPF) (Dunphy et al., 1988; Gautier et al., 1988), which plays an essential role in the entry to mitosis (Nurse, 1990). A closely related kinase subunit, p33\textsuperscript{cdk2} (Elledge and Spottswood, 1991; Hirai et al., 1992; Ninomiya-Tsuji et al., 1991; Paris et al., 1991; Tsai et al., 1991), is thought to function earlier in the cell cycle than p34\textsuperscript{cdc2} (Dulić et al., 1992; Koff et al., 1991; Koff et al., 1992; Lees et al., 1992). In mammalian cells, p33\textsuperscript{cdk2} associates with cyclin E in G\textsubscript{1}-phase (Dulić et al., 1992; Koff et al., 1992) and cyclin A during S-phase (Rosenblatt et al., 1992). A role for p33\textsuperscript{cdk2} has also been suggested in CSF-induced M-phase arrest (Gabrielli et al., 1993).

The activity of p34\textsuperscript{cdc2} depends on association with a cyclin subunit but is also regulated by phosphorylation and dephosphorylation on p34\textsuperscript{cdc2} (reviewed by Murray, 1993). Phosphorylation of residues T14 and Y15 of p34\textsuperscript{cdc2} inhibit its activity, whereas phosphorylation of the T161/T160 residues of p34\textsuperscript{cdc2}/p33\textsuperscript{cdk2} abolishes the activation of p34\textsuperscript{cdc2} (reviewed by Murray, 1993). Phosphorylation of T161/T160 may induce a change in the conformation of the T-loop and allow access of the substrate. The T161/T160 is phosphorylated by a kinase termed CAK (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1992) as is the phospho-antagonistic roles. p33\textsuperscript{cdk2} may also be regulated in a similar manner to p34\textsuperscript{cdk2} (Gabrielli et al., 1992; Gu et al., 1992; Sebastian et al., 1993). Conversely, phosphorylation of T161 is required for the activation of p34\textsuperscript{cdc2} (Gould et al., 1991; Norbury et al., 1991; Solomon et al., 1992) as is the phosphorylation of T160 in p33\textsuperscript{cdk2} (Gu et al., 1992; Poon et al., 1993). The T161/T160 is phosphorylated by a kinase termed CAK, an important question is whether its activity is regulated during progression through the cell cycle, since p40\textsuperscript{MO15} is necessary (albeit not sufficient) for the activation of both p34\textsuperscript{cdc2} and p33\textsuperscript{cdk2}. Any variation of p40\textsuperscript{MO15} activity in the cell cycle could potentially play a role in the regulation of p34\textsuperscript{cdc2}/p33\textsuperscript{cdk2}. In this paper, we show that the level and activity of p40\textsuperscript{MO15} in cell extracts are constant during the rapid cell cycles of embryonic cells and synchronised cultured cells, but both the level and activity of p40\textsuperscript{MO15} are diminished when mouse Swiss 3T3 cells enter quiescent G\textsubscript{0}-phase as a result of serum deprivation; under these conditions, the levels of p34\textsuperscript{cdc2}/p33\textsuperscript{cdk2} and of most of the cyclins are reduced at least as much, if not more.

Given that p40\textsuperscript{MO15} is a component of CAK, an important question is whether its activity is regulated during progression through the cell cycle, since p40\textsuperscript{MO15} is necessary (albeit not sufficient) for the activation of both p34\textsuperscript{cdc2} and p33\textsuperscript{cdk2}. Any variation of p40\textsuperscript{MO15} activity in the cell cycle could potentially play a role in the regulation of p34\textsuperscript{cdc2}/p33\textsuperscript{cdk2}. In this paper, we show that the level and activity of p40\textsuperscript{MO15} in cell extracts are constant during the rapid cell cycles of embryonic cells and synchronised cultured cells, but both the level and activity of p40\textsuperscript{MO15} are diminished when mouse Swiss 3T3 cells enter quiescent G\textsubscript{0}-phase as a result of serum deprivation; under these conditions, the levels of p34\textsuperscript{cdc2}/p33\textsuperscript{cdk2} and of most of the cyclins are reduced at least as much, if not more.
We demonstrated previously that a bacterially expressed glutathione S-transferase (GST)-MO15 fusion protein could be activated by ‘marinating’ it in crude cell extracts (Poon et al., 1993). Although the level and activity of endogenous p40MO15 did not vary in the cell cycle, we found that GST-MO15 was more rapidly and completely activated by M-phase cell extracts than by cell extracts in interphase. GST-MO15 was phosphorylated on both serine and threonine by these extracts, and the rate of phosphorylation of GST-MO15 was higher in M-phase extracts than in interphase extracts. Mutation studies suggested that one of the serine phosphorylation sites in GST-MO15 is serine 170 (a p34cdc2/p33cdk2 phosphorylation site), but mutation of S170 to alanine did not affect the activity of GST-MO15. By contrast, mutation of T176 to alanine completely inhibited the ability of GST-MO15 to acquire protein kinase activity.

**MATERIALS AND METHODS**

**Plasmid constructs**

Histidine-tagged Protein A-cyclin A (PA-cyclin A), GST-cdk2, GST-cdk2 K33R, GST-MO15 and GST-MO15 K47R constructs were described previously by Poon et al. (1993). The T176A, S170A and S186A mutants of GST-MO15 were constructed by PCR essentially as described by Horton and Pease (1991) with the following oligonucleotides and their antisense counterparts:

T176A, 5′ CTGACTTACGCCCATGAGGTG 3′; S170A, 5′ AAGTCAATGTTGGGCCCACAAGAGATA 3′; S186A, 5′ TGGTACCGCCTCTGAGTTG 3′.

The mouse MO15 clone (whose sequence is listed under GenBank accession number X74145) described by Ershler et al. (1993) and Stepanova et al. (1994) was amplified by PCR with the following oligonucleotides: 5′ CCTGGAGATTCAGATCTTGAT 3′ and 5′ CTTTGGATCTTTTATTTTACG 3′. The amplified fragment was cut with BglII and BamHI and ligated with BamHI-cut pET16b vector. This construct expressed the C-terminal 130 residues of mouse MO15 with an N-terminal decahistidine ‘tag’ when induced with IPTG. By contrast, mutation of T176 to alanine completely inhibited the ability of GST-MO15 to acquire protein kinase activity.

**Expression and purification of recombinant proteins from bacteria**

PA-cyclin A was expressed in *Escherichia coli* and purified by NTA-Ni2+ agarose (Qiagen) affinity chromatography as described previously (Poon et al., 1993) except that EDTA was omitted from the bacterial lysis buffer (see Poon and Hunt, 1994). Wild-type or mutant GST-MO15 and GST-cdk2 were expressed in *E. coli* and purified by affinity chromatography on GSH-Sepharose as described previously (Poon et al., 1993).

**Preparation of Xenopus egg extracts**

Xenopus CSF-arrested extracts were prepared following the method of Murray (1991); 100 μg/ml of cycloheximide was added so that CSF-arrested extracts contained the same concentration of cycloheximide as interphase extracts. Interphase extracts were prepared by making CSF-arrested extracts 0.4 mM in CaCl2 and 100 μg/ml cycloheximide, followed by incubation at 23°C for 20 minutes. Interphase extracts were subsequently ‘activated’ if required by addition of 100 μg/ml PA-cyclin A (1.42 μM) and incubation at 23°C for 20 minutes.

**Xenopus developmental samples**

Freshly laid Xenopus eggs were fertilised in 0.5 mM HEPES, pH 7.8, 8.8 mM NaCl, 0.1 mM KCl, 0.07 mM CaCl2, 0.1 mM MgSO4, 0.25 mM NaHCO3. At different times after fertilisation, five embryos at the same stage were sampled and frozen in liquid nitrogen. Embryos were staged according to Nieuwkoop and Faber (1994). The samples were mixed with 300 μl of SDS-sample buffer and boiled. Insoluble material was removed by centrifugation for 5 minutes in a microfuge.

**Synchronisation of mouse Swiss 3T3k cells**

Swiss 3T3k cells were grown in 15 cm plates in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) foetal calf serum in a humidified incubator at 37°C, in 5% CO2 until the cultures reached about 50% confluency. The medium was then changed to DMEM with 0.2% (v/v) foetal calf serum for 48 hours. Cells were released from the Go arrest by supplying the cells with medium containing DMEM with 10% (v/v) foetal calf serum. Samples were taken at 3 hour intervals for 27 hours after ‘refeeding’. At each time point, the cells were washed with phosphate buffered saline (137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) and scraped off the plate. One fifth of the sample was resuspended in 70% ethanol for later FACS (fluorescence-activated cell sorter) analysis. The rest of the cells were harvested by centrifugation and mixed with twice the pellet volume of a detergent lysis buffer containing 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.2% Nonidet P-40, 10 μg/ml cytochalasin B, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 15 μg/ml benzamidine, 10 μg/ml chymostatin, 10 μg/ml pepstatin, 1 mM PMFS, 10 μg/ml soybean trypsin inhibitor. The suspension was incubated on ice for 30 minutes and cell debris removed by centrifugation in a microfuge at 4°C for 30 minutes. The protein concentration of the cell extract was measured with the Bradford dye method (Bradford, 1976).

**Histone H1 kinase assay**

The histone H1 kinase activity of cell extracts was measured by mixing 1 μl of extract with 9 μl of kinase buffer (80 mM Na-β-glycero phosphate, pH 7.4, 20 mM EGTA, 15 mM Mg(OAc)2 and 1 mM DTT) supplemented with 1 μg histone H1, 30 μM ATP and 1.25 μCi [γ-32P]ATP followed by incubation at 23°C for 20 minutes. The reaction was terminated by addition of 25 μl of SDS-sample buffer and analysed by SDS-PAGE and autoradiography. For assaying immobilised samples, the beads were mixed with 10 μl of kinase buffer supplemented with 1 μg histone H1, 30 μM ATP and 1.25 μCi [γ-32P]ATP, and then the same procedure as described above was followed.

**GST-cdk2 kinase assay**

Immunoprecipitates of p40MO15 or immobilised GST-MO15 were washed three times with 250 μl of bead buffer (50 mM Tris-HCl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 2 μg/ml aprotinin, 15 μg/ml benzamidine, 1 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor) and twice with 250 μl of kinase buffer. The kinase activity towards GST-cdk2 was measured by adding 10 μl of kinase buffer containing 30 μM ATP, 5 μCi [γ-32P]ATP and 100 μg/ml GST-cdk2 K33R. The reaction was carried out at 23°C for 30 minutes and terminated by addition of 25 μl of SDS-PAGE sample buffer. The samples were analysed by SDS-PAGE and autoradiography.

**Activation of GST-MO15 by cell extracts**

Xenopus egg extract (10 μl) was incubated with 100 μg/ml of GST-MO15 (or mutants) at 23°C for 30 minutes. The sample was then mixed with 250 μl of bead buffer, and GST-MO15 was harvested with 10 μl of GSH-Sepharose with end-to-end rotation at 4°C for 30 minutes. The beads were washed three times with 250 μl of bead buffer and twice with kinase buffer. The protein kinase activity retained on the beads using GST-cdk2 K33R as substrate was then assayed as described above.
Phosphorylation of GST-MO15 by cell extracts

*Xenopus* egg extract was diluted five-fold with kinase buffer, 0.5 µCi/µl [γ-32P]ATP and 100 µg/ml of GST-MO15 (or mutant variants thereof) were added, and the mixture incubated at 23°C for 60 minutes. The reaction was terminated by addition of 250 µl of ice-cold bead buffer, and GST-MO15 was harvested with 10 µl of GSH-Sepharose with end-to-end rotation at 4°C for 30 minutes. The beads were washed five times with 250 µl of bead buffer. The GST-MO15 was eluted from the beads with 25 µl of SDS-sample buffer and analysed by SDS-PAGE and autoradiography.

**Antibodies and immunological methods**

An anti-mouse p40MO15 antiserum was raised in rabbits by subcutaneous injection of inclusion bodies of bacterially expressed mouse MO15 fragment (the C-terminal 130 amino acids). Rabbit anti-cyclin A antibody and anti-cyclin B1 antibody were raised against hamster cyclin A and cyclin B1, respectively (Tazunoki et al., unpublished data). These antibodies also recognize mouse cyclin A and cyclin B1, respectively. Rabbit anti-*Xenopus* p40MO15 antiserum was raised against a synthetic peptide corresponding to the C-terminal 11 amino acids of *Xenopus* p40MO15 (CADQDIKAKL). Rabbit anti-GST antiserum was as described by Poon et al. (1993). Rabbit anti-human p33cdc2 antiserum was raised against a synthetic peptide corresponding to the C-terminal 15 amino acid residues of human p33cdc2 (PFFQDVTKPVPHLRL); this antiserum also recognizes mouse p33cdc2. Rabbit anti-*Xenopus* p33cdc2 antiserum was raised against bacterially expressed *Xenopus* p33cdc2. Anti-p34cdc2 monoclonal antibody (A17) was raised against the C-terminal half of *Xenopus* p34cdc2 as described by Kobayashi et al. (1991); this antibody also recognizes mouse and human p34cdc2. Immunoblotting and immunoprecipitation were performed as described previously (Poon et al., 1993).

**RESULTS**

**Cell cycle variation of p40MO15 level and activity**

To study the level and activity of p40MO15 during *Xenopus* rapid cleavage cell cycles, *Xenopus* CSF-arrested extract (with high histone H1 kinase activity) was induced to enter interphase (with low histone H1 kinase activity) by addition of 0.4 mM CaCl2. Cycloheximide was added to prevent further cyclin synthesis, which would otherwise allow the extract eventually to return to M-phase spontaneously. The histone H1 kinase activity of the extract was returned to a high level by addition of 1.4 µM Protein A-cyclin A (PA-cyclin A) protein (Fig. 1, top panel). The relative amount of p40MO15 in the cell extracts was measured by immunoblotting with anti-p40MO15 antibody (middle panel; note that the Protein A moiety on the cyclin A fusion protein reacts with the second antibody used for ECL detection). The CAK activity of p40MO15 was measured by the ability of p40MO15 immunoprecipitates to phosphorylate GST-cdk2 (bottom panel). Fig. 1 shows that both the level of p40MO15 and its activity were the same in M-phase and interphase extracts.

**Phosphoamino-acid analysis**

One-dimensional phosphoamino-acid analysis after partial acid hydrolysis was performed on 32PO4-labelled polypeptides after transfer to Immobilon (Millipore) as described by Kamps (1991). Polygram TLC plates (Macherey-Nagel) were used to separate phosphoamino acids by electrophoresis at 1000 V for 30 minutes with pH 3.5 buffer. Samples for analysis were mixed with phosphoserine, phosphothreonine and phosphotyrosine standards and stained with ninhydrin prior to autoradiography to determine their mobility.

**Xenopus** somatic cultured cells (WAK cells) can be blocked at the G1/S boundary with aphidicolin, and cells can be released synchronously into the cell cycle by removal of aphidicolin. No change in the level or activity of p40MO15 was found in WAK cells after release from the aphidicolin block (data not shown).

To study p40MO15 activity in somatic cell cycles in more detail, we raised an antiserum against the bacterially expressed C-terminal half of mouse p40MO15 (Stepanova et al., 1994). The anti-mouse p40MO15 antiserum recognised a 40 kDa protein in immunoblots of Swiss 3T3K cell extracts, but did not recognise the p40MO15 from a similar amount of human HeLa cell extract or *Xenopus* WAK cell extract (Fig. 2A). Fig. 2B, lane 2, shows that the anti-mouse p40MO15 antibody also immunoprecipitated GST-cdk2 kinase activity from Swiss 3T3K cell extract, whereas preimmune serum (lane 1) did not.

On serum starvation, the mouse cell line Swiss 3T3K (Todaro and Green, 1963) exit the cell cycle to a quiescent state (G0), and can be released from G0 back into the cell cycle by addition of serum. Swiss 3T3K cells were arrested by growing in medium containing 0.2% (v/v) foetal calf serum for 48 hours and the cells were then released by supplying the cells with medium containing 10% foetal calf serum. Cells were sampled at 3 hour time points for FACS analysis and cell-free extracts. FACS analysis revealed that more than 85% of the cells had a 2C DNA content after serum starvation (Fig. 3A). The serum-stimulated cells started to enter S-phase about 15 hours after release from serum starvation, indicating that the cells had been arrested in G0 rather than G1-phase. Almost
immunoblotted with anti-mouse p40 MO15 antibody, and the
Dynamics).

of GST-cdk2 was detected with a phosphorimager (Molecular

human HeLa cells (lane 2) and

µ

Xenopus

anti-p40 MO15 antiserum (Fig. 3C) showed that the level
activity and the level of endogenous p40 MO15 did not vary

all the cells were in S-phase 18 hours after serum release; they
reached G2-phase at around 21 hours and completed M-phase
by 24 hours.

The protein concentrations of the cell extracts were measured and adjusted to the same concentration with buffer.

After immunoprecipitation with anti-p40 MO15 antiserum, the kinase activity of each sample was assayed using kinase-dead
GST-cdk2 K33R as substrate. Fig. 3B shows that the CAK activity associated with p40 MO15 immunoprecipitates from Go-arrested
was about 10-15% of that in exponentially growing cells, and that the level of CAK activity increased gradually
while the cells re-entered the cell cycle to G1-phase. The
activity stayed relatively constant thereafter. Immunoblotting with anti-p40 MO15 antiserum (Fig. 3C) showed that the level
of p40 MO15 was also reduced by about 90% in Go cells and
increased after serum stimulation in parallel with the profile of
p40 MO15 activity shown in Fig. 3J. The same samples were also
immunoblotted with anti-p34 cdc2, p33 cdk2, cyclin A and cyclin
B1 antibodies (Fig. 3D,E,F and G). The level of p33 cdk2
was undetectable in Go cells and did not begin to
increase until 11 hours after serum stimulation. Cyclin A was
first detectable at about 11 hours and cyclin B at 15 hours after
stimulation, rising to a peak at 21 hours in late G2- to
M-phase, with cyclin A rising 1-2 hours earlier than cyclin B.
The levels of cyclin A and cyclin B were closely related to the
histone H1 kinase activity associated with p33 cdk2 and p34 cdc2,
respectively (Fig. 3H and I). Fig. 3I top panel shows the quan-
tification of the GST-cdk2 kinase activity associated with
p40 MO15, and the histone H1 kinase activity associated with
p34 cdc2 and p33 cdk2 (from Fig. 3B,H and I). Fig. 3J middle
and bottom panels shows the quantitation of the amount of
p40 MO15, p34 cdc2, p33 cdk2, cyclin A and cyclin B1 from Fig.
3C to G. It is noteworthy that the levels of p34 cdc2 and of cyclins A and B1 fell below the level of detection during serum
starvation, whereas the levels of p40 MO15 and of p33 cdk2 were
maintained at about 15% and 25% (respectively) of their levels in
exponentially growing (and cycling) cells.

p40 MO15 during Xenopus early development

The levels of p40 MO15 during early development of Xenopus embryos were measured by immunoblotting samples of
Xenopus embryos with anti-p40 MO15 antibody. Equal numbers of embryos were taken at different stages of development up
to the tadpole stage with tails about 1 cm long, as indicated in
Fig. 4. Immunoblotting with anti-p40 MO15 antibody revealed a
low but detectable level of p40 MO15 that decreased slightly
during the early rapid cell divisions, increased around gastrula
stage and then stayed relatively constant up to the tadpole stage
(Fig. 4, top panel). In comparison, the level of p34 cdc2
remained at a roughly constant level after a transient decline
during the early rapid cell divisions (Fig. 4, middle panel). The
level of p33 cdk2 was about one-tenth of that of p34 cdc2 in unfer-
tilised eggs and its amount also decreased slightly during the
early rapid cell divisions, then slowly increased after gastrula
stage, becoming comparable with that of p34 cdc2 in swimming
tadpoles (Fig. 4, bottom panel). The concentration of p34 cdc2
in egg extracts lies between 0.5 and 1 µM (E. Stewart, personal
communication).

Bacterially expressed MO15 is more rapidly activated by M-phase cell extracts than by interphase cell extracts

As we reported previously, GST-MO15 fusion protein produced in bacteria was inactive as a protein kinase, but it
acquired activity after incubation in Xenopus egg extract (Poon et al., 1993). We next compared the ability of M-phase or inter-
phase extracts to activate GST-MO15. As shown in Fig. 1, the
activity and the level of endogenous p40 MO15 did not vary between M-phase egg extracts and interphase egg extracts. We
were therefore surprised to find that bacterially-expressed
GST-MO15 was more strongly activated by M-phase frog egg
extracts than by interphase egg extracts (Fig. 5A). GST-MO15
was added to CSF-arrested egg extract, interphase egg extract or interphase egg extract reactivated with PA-cyclin A. After
incubation, the GST-MO15 was recovered by affinity chroma-
tography on GSH-Sepharose and assayed for its kinase activity using kinase-dead GST-cdk2 as a substrate. GST-
MO15 was less active as a GST-cdk2 kinase after incubation with
interphase extract (lane 3) than with CSF-arrested extract
(lane 2) or interphase extract treated with PA-cyclin A (lane 4).
Fig. 5B shows that the rate of activation of GST-MO15 by the
M-phase extract was at least 5-fold higher than the rate of
activation in interphase extract. The reason for this difference
remains to be explored further, but we speculate that either
there is a higher concentration of (putative) p40 MO15 partner(s)
in M-phase extracts, or that the level of p40 MO15-activating
protein kinase is higher in M-phase than in interphase extracts
(see Discussion). This result prompted us to investigate
whether GST-MO15 was phosphorylated by cell extracts.
Fig. 3. p40MO15 in synchronised Swiss 3T3K cells. (A) Swiss 3T3K cells were synchronised by serum starvation as described in the text. About 5×10⁶ cells were taken at the indicated time points, fixed in 70% ethanol and stained with propidium iodide prior to FACS analysis. The vertical axis indicates the relative frequency of cells and the horizontal axis indicates the DNA content of the cells at the indicated time points after serum starvation. (B) Cell extracts were made from cells sampled at the indicated times and adjusted to the same protein concentration. The cell extracts were immunoprecipitated with anti-p40MO15 antibody and the GST-cdk2 kinase activity was assayed. The cell extracts were dissolved in SDS-sample buffer and immunoblotted with (C) anti-p40MO15 antibody; (D) anti-p34cdc2 antibody (A17); (E) anti-p33cdk2 antibody; (F) anti-cyclin A antibody (E23); and (G) anti-cyclin B1 antibody (from the same immunoblot after probing for cyclin A, without stripping the anti-cyclin A antibody). The histone H1 kinase activity associated with (H) p34cdc2 and (I) p33cdk2 immunoprecipitates were assayed as described in Materials and Methods. (J) Quantitation of the GST-cdk2 kinase activity associated with p40MO15 and the histone H1 kinase activity associated with p34cdc2 and p33cdk2 (top panel), the amount of p40MO15, p34cdc2, p33cdk2 (middle panel), cyclin A and cyclin B1 (bottom panel).
When phosphorylation reactions in a *Xenopus* egg extract were inhibited by addition of EDTA to chelate Mg$^{2+}$ and other divalent metal ions, the activation of GST-MO15 as a kinase for GST-cdk2 was completely inhibited (Fig. 6A, lane 4). A kinase-inactive mutant of GST-MO15 with the lysine in the ATP-binding region mutated to arginine (K47R) cannot be activated by cell extracts (lanes 5 and 6). The ATP-dependence of the activation, and the analogy with the activation of p33 cdk2 and p34 cdc2 suggested that phosphorylation was involved in the activation of GST-MO15.

We tested whether GST-MO15 was phosphorylated by cell extracts by mixing GST-MO15 K47R (which is incapable of self-phosphorylation) and $[^\gamma-32\text{P}]$ATP with *Xenopus* egg extract. After incubation, the GST-MO15 K47R was recovered with glutathione (GSH)-Sepharose and its phosphorylation was detected using SDS-PAGE and autoradiography. Under these conditions, GST-MO15 K47R was phosphorylated (Fig. 6B, lane 1). Phosphoamino-acid analysis of the phosphorylated band revealed mainly phosphoserine, although some phosphorylation on threonine was detectable, particularly if okadaic acid was present to inhibit protein phosphatase activity (lane 2). Digestion with thrombin revealed that the phosphorylation of GST-MO15 was on the MO15 domain and not on the GST domain (data not shown).

To test whether there was any cell cycle variation in the rate of phosphorylation of p40$^{\text{MO15}}$, GST-MO15 was incubated with *Xenopus* CSF-arrested egg extract, interphase egg extract, and early neurula extract. After incubation, the GST-MO15 K47R was recovered with glutathione (GSH)-Sepharose and its phosphorylation was detected using SDS-PAGE and autoradiography. Under these conditions, GST-MO15 K47R was phosphorylated (Fig. 6B, lane 1). Phosphoamino-acid analysis of the phosphorylated band revealed mainly phosphoserine, although some phosphorylation on threonine was detectable, particularly if okadaic acid was present to inhibit protein phosphatase activity (lane 2). Digestion with thrombin revealed that the phosphorylation of GST-MO15 was on the MO15 domain and not on the GST domain (data not shown).
or interphase egg extract treated with PA-cyclin A. As expected, the histone H1 kinase activity was at a high level in CSF-arrested egg extract and cyclin A-treated egg extract, but at a much reduced level in interphase extract (Fig. 6C, top panel). Phosphorylation of GST-MO15 (Fig. 6C, middle panel) was likewise higher in CSF-arrested egg extract (lane 1) than in interphase egg extract (lane 2), and the level of phosphorylation was very significantly elevated in interphase extract treated with cyclin A (lane 3).

Given that the GST-MO15 was phosphorylated mainly on serine residues, and the phosphorylation was higher when the kinase activity of p34<sup>cdc2</sup>/p33<sup>cdk2</sup> was activated (in CSF-arrested egg extract and cyclin A-treated extract), it was possible that GST-MO15 was directly phosphorylated by p34<sup>cdc2</sup>/p33<sup>cdk2</sup>. The cyclin-dependent kinases p34<sup>cdc2</sup> and p33<sup>cdk2</sup> have a relatively rigid phosphorylation site specificity of a serine or threonine residue followed by a proline. *Xenopus* p40<sup>MO15</sup> contains two putative serine phosphorylation sites for p34<sup>cdc2</sup>/p33<sup>cdk2</sup> at S170 and S186. Among p40<sup>MO15</sup> from *Xenopus*, goldfish, mouse and human, S170 is present in all four species, but S186 is only present in *Xenopus*. Two mutants of *Xenopus* GST-MO15 were therefore constructed, with S170 or S186 changed to non-phosphorylatable alanine residues (S170A and S186A, respectively). These mutants, together with GST-MO15, GST-MO15 K47R, and GST-MO15 T176A (T176 is the equivalent residue to T160 in p33<sup>cdk2</sup>) were tested for their ability to be phosphorylated in *Xenopus* egg extract (Fig. 7A). Wild-type GST-MO15, K47R and T176A mutants were all phosphorylated by egg extract to similar extents (top panel, lanes 2-4), the phosphorylation was predominantly on serine and only weakly on threonine (bottom panel). But GST-MO15 S170A was phosphorylated significantly less than the other GST-MO15 proteins (lane 5), although immunoblotting confirmed that a similar amount of protein was present in the reactions (middle panel). Phosphoamino-acid analysis revealed that most (but not all) serine phosphorylation seen in GST-MO15 was removed in the S170A mutant, whereas the threonine phosphorylation was not affected. No significant effect on the phosphorylation of GST-MO15 was detected with the S186A mutant (lane 6). We also found that GST-MO15 S170A could be phosphorylated by cyclin B·p34<sup>cdc2</sup>·immunoprecipitates in vitro, and that mutation of S170A abolished most of this phosphorylation (data not shown). More surprisingly, however, mutation of T176 to alanine did not abolish the phosphothreonine labelling in this procedure.

To test whether any of the mutations of GST-MO15 shown above affected their CAK activity, GST-MO15 and its mutants were incubated with cell extracts, harvested with GSH-Sepharose and assayed for their kinase activities using GST-cdk2 as a substrate. Fig. 7B shows that the K47R and T176A mutations abolished the GST-cdk2 kinase activity of GST-MO15 (lanes 2-4). Longer exposure of the autoradiograph
showed that the T176A mutant retained very low GST-cdk2 kinase activity, whereas no such activity was detectable with the K47R mutant. This is reminiscent of the properties of p33cdc2, which retains about 1% of its kinase activity when T160 is changed to alanine (Connell-Crowley et al., 1993). By contrast, the GST-cdk2 kinase activities of the S170A and S186A mutants were essentially the same as that of wild-type GST-MO15 (Fig. 7B, lanes 5 and 6). Thus, although S170 is clearly phosphorylated by cell extracts containing active p34cdc2/p33cdc2 kinase, mutation of S170 to alanine does not significantly affect the kinase activity of GST-MO15, and the rate of activation of the S170A construct in mitotic extracts was essentially the same as that of the wild-type construct (data not shown).

**DISCUSSION**

**Cell cycle variation of p40MO15 activity**

We show in this paper that there is no substantial variation on the level or kinase activity of p40MO15 (CAK) during progress through either embryonic or somatic cell cycles. Similar conclusions have been reached by Tassan et al. (1994). Only when cells are induced to exit the cell cycle to a G0 state by serum deprivation do the level and the kinase activity of p40MO15...
become diminished. It is unlikely that the decrease in activity of p40MO15 in G0 is important in reducing the activity of either p34cdc2 or p33cdk2, however, since the levels of both kinase subunits are themselves reduced by serum starvation. More critically, there was no detectable cyclin A nor cyclin B in extracts of serum-starved cells, and the lack of histone H1 kinase activity associated with p34cdc2 and p33cdk2 in G0 can readily be ascribed to the combined lack of p34cdc2, p33cdk2 and cyclins, rather than of p40MO15. These results do not exclude the possibility that p40MO15 might be regulated in the cell cycle by other means, such as subcellular compartmentation. It is also possible that the p40MO15 activity varies in the normal cell cycle in a way that our assay fails to register, and this variation might be sufficient to affect the activity of CDKs. It is also possible that the activity of p40MO15 towards substrates other than p34cdc2/p33cdk2 may vary in the cell cycle, although at present, no such substrates have been identified.

**Phosphorylation regulation of p40MO15**

As discussed above, the activation of GST-MO15 by cell extracts is suspected to involve binding of GST-MO15 to a partner and phosphorylation of GST-MO15. Here we show that GST-MO15 is phosphorylated by cell extracts on serine and threonine residues. One phosphorylation site is likely to be S170, but mutation of S170 to alanine did not impair the activity of GST-MO15. On the other hand, mutation of T176 to alanine strongly inhibited GST-MO15 activation by cell extracts. Therefore, it is likely, although not rigorously proven that, as in the activation of p34cdc2, phosphorylation of T176 of p40MO15 is necessary for its activity. It will be very interesting to learn what kinase is responsible for the phosphorylation of T176.

Mutation of T176 to alanine (T176A) did not affect the extent of GST-MO15 phosphorylated by cell extracts (Fig. 7A). Moreover, T176A mutant did not appear to reduce the small amount of threonine phosphorylation detected in GST-MO15, indicating that T176A mutant indicates that T176 is important for the activity of GST-MO15 (Fig. 7B). We suspect that T176 is phosphorylated in the active form of GST-MO15, and that the apparent weak phosphorylation detectable on T176 may be due to the fact that GST-MO15 may need to bind to a partner(s) in the cell extract before it can be phosphorylated on T176, and the partner(s) available for GST-MO15 may be limited in the cell extracts we used. On the other hand, phosphorylation of residues like S170 may not require association with partner(s) and is therefore much stronger. We suspect that only a small fraction of the added bacterially-produced GST-MO15 is converted to the enzymically active form.

**Phosphorylation of S170**

As discussed above, the activation of GST-MO15 by cell extracts is suspected to involve binding of GST-MO15 to a partner and phosphorylation of GST-MO15. Here we show that GST-MO15 is phosphorylated by cell extracts on serine and threonine residues. One phosphorylation site is likely to be S170, but mutation of S170 to alanine did not impair the activity of GST-MO15. On the other hand, mutation of T176 to alanine strongly inhibited GST-MO15 activation by cell extracts. Therefore, it is likely, although not rigorously proven that, as in the activation of p34cdc2, phosphorylation of T176 of p40MO15 is necessary for its activity. It will be very interesting to learn what kinase is responsible for the phosphorylation of T176.

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**Phosphorylation of S170**

As discussed above, the activation of GST-MO15 by cell extracts is suspected to involve binding of GST-MO15 to a partner and phosphorylation of GST-MO15. Here we show that GST-MO15 is phosphorylated by cell extracts on serine and threonine residues. One phosphorylation site is likely to be S170, but mutation of S170 to alanine did not impair the activity of GST-MO15. On the other hand, mutation of T176 to alanine strongly inhibited GST-MO15 activation by cell extracts. Therefore, it is likely, although not rigorously proven that, as in the activation of p34cdc2, phosphorylation of T176 of p40MO15 is necessary for its activity. It will be very interesting to learn what kinase is responsible for the phosphorylation of T176.

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**Phosphorylation of S170**

As discussed above, the activation of GST-MO15 by cell extracts is suspected to involve binding of GST-MO15 to a partner and phosphorylation of GST-MO15. Here we show that GST-MO15 is phosphorylated by cell extracts on serine and threonine residues. One phosphorylation site is likely to be S170, but mutation of S170 to alanine did not impair the activity of GST-MO15. On the other hand, mutation of T176 to alanine strongly inhibited GST-MO15 activation by cell extracts. Therefore, it is likely, although not rigorously proven that, as in the activation of p34cdc2, phosphorylation of T176 of p40MO15 is necessary for its activity. It will be very interesting to learn what kinase is responsible for the phosphorylation of T176.

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GST-MO15 in our assays, it is possible that S170 phosphorylation has an effect on the activity of p40\(^{MO15}\) that was not detectable in the in vitro assay we used. Furthermore, S170 phosphorylation might be required for other processes, like nuclear localisation (Tassan et al., 1994), which we did not explore.

The result that GST-MO15 is activated more rapidly and completely by M-phase extract than by interphase extract (Fig. 5) is peculiar, since the activity of the endogenous p40\(^{MO15}\) does not vary in these cell extracts (Fig. 1). One possibility suggested by this result is that the activating kinase for p40\(^{MO15}\) (possibly for T176) may be more active in M-phase, in which case the endogenous p40\(^{MO15}\) would not be affected because it is already phosphorylated (assuming the turnover of phosphorylated T176 is slow). Alternatively, the activity of the phosphatase for T176 of p40\(^{MO15}\) may be lower in M-phase extracts than in interphase extracts. Another possibility is that phosphatase for T176 of p40\(^{MO15}\) may be lower in M-phase than in interphase extracts, or that there are more putative partner subunits available for GST-MO15 in M-phase extracts than in interphase extracts. Finally, it may be that the bacterially-expressed GST-MO15 requires significant refolding by chaperone-like proteins after it has been added to the extracts, and that the activity of these entities is different under different conditions. We cannot easily distinguish between these possibilities until the partner subunit(s) of p40\(^{MO15}\) have been identified, which would allow the design and execution of more precisely focused biochemical assays.

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