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

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SUMMARY

A key component of Cdc2/Cdk2-activating kinase (CAK) is p40^{MO15}, a protein kinase subunit that phosphorylates the T161/T160 residues of p34^{cdc2}/p33^{cdk2}. The level and activity of p40^{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^{MO15}. Although the level and activity of endogenous p40^{MO15} did not vary in the cell cycle, we found that bacterially expressed p40^{MO15} was activated more rapidly by M-phase cell extracts than by interphase cell extracts. Bacterially expressed p40^{MO15} was phosphorylated mainly on serine 170 (a p34^{cdc2} phosphorylation site) by mitotic cell extracts, but mutation of S170 to alanine did not affect the activation of p40^{MO15}, whereas mutation of T176 (the equivalent site to T161/T160 in p34^{cdc2}/p33^{cdk2}) abolished the activation of p40^{MO15}. These studies suggest that the level and activity of p40^{MO15} is probably not a major determinant of p34^{cdc2}/p33^{cdk2} activity in the cell cycle, and that the activation of p40^{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_1/S-phase in conjunction with different cyclin subunits. Cyclin B and p34^{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^{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^{cdc2} (Dulić et al., 1992; Koff et al., 1991; Koff et al., 1992; Lees et al., 1992). In mammalian cells, p33^{cdk2} associates with cyclin E in G_1-phase (Duli´c et al., 1992; Koff et al., 1992) and cyclin A during S-phase (Rosenblatt et al., 1992). A role for p33^{cdk2} has also been suggested in CSF-induced M-phase arrest (Gabrielli et al., 1993).

The activity of p34^{cdc2} depends on association with a cyclin subunit but is also regulated by phosphorylation and dephosphorylation on p34^{cdc2} (reviewed by Murray, 1993). Phosphorylation of residues T14 and Y15 of p34^{cdc2} inhibit its activity, where the Wee1 protein kinase (Featherstone and Russell, 1991; Parker et al., 1992; Russell and Nurse, 1987) and Cdc25 tyrosine phosphatases (Dunphy and Kumagai, 1991; Gautier et al., 1991; Lee et al., 1992; Millar et al., 1991) have mutually antagonistic roles. p33^{cdk2} may also be regulated in a similar manner to p34^{cdc2} (Gabrielli et al., 1992; Gu et al., 1992; Sebastian et al., 1993). Conversely, phosphorylation of T161 is required for the activation of p34^{cdc2} (Gould et al., 1991; Norbury et al., 1991; Solomon et al., 1992) as is the phosphorylation of T160 in p33^{cdk2} (Gu et al., 1992; Poon et al., 1993). The T161/T160 is phosphorylated by a kinase termed p34^{cdc2}/p33^{cdk2}-activating kinase (CAK), and the CDK-related protein p40^{MO15} was recently identified as the catalytic subunit of CAK (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). Structural studies of p34^{cdc2}/p33^{cdk2} suggest that phosphorylation of T161/T160 may induce a change in the conformation of the T-loop and allow access of the substrate to ATP (De Bondt et al., 1993). It is clear that like other members of the cdc2 family of protein kinases, p40^{MO15} requires additional protein subunits for activity, but at present their number and identity is not clear. In starfish, the active enzyme comprises two components (Fesquet et al., 1993), whereas there is recent evidence that human CAK may have three subunits (Tassan et al., 1994).

Given that p40^{MO15} is a component of CAK, an important question is whether its activity is regulated during progression through the cell cycle, since p40^{MO15} is necessary (albeit not sufficient) for the activation of both p34^{cdc2} and p33^{cdk2}. Any variation of p40^{MO15} activity in the cell cycle could potentially play a role in the regulation of p34^{cdc2}/p33^{cdk2}. In this paper, we show that the level and activity of p40^{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^{MO15} are diminished when mouse Swiss 3T3K cells enter quiescent G_0-phase as a result of serum deprivation; under these conditions, the levels of p34^{cdc2}/p33^{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 $p40^{MO15}$ 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 $p34^{cdc2}/p33^{cdk2}$ 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 a PCR method essentially as described by Horton and Pease (1991) with the following oligonucleotides and their antisense counterparts:

T176A, 5′ CGTACTTACGCCCATGAGGTG 3′;
S170A, 5′ AAGTCATTTGGGGCCCCAAACAGAATA 3′;
S186A, 5′ TGGTACCGAGCTCCTGAGTTG 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′ CCTTTGGATCCTTTATTTCAG 3′. The amplified fragment was cut with *Bgl*II and *Bam*HI and ligated with *Bam*HI-cut pET16b vector. This construct expressed the C-terminal 130 residues of mouse MO15 with an N-terminal decahistidine 'tag' when induced with IPTG (isopropyl β-D-thiogalactopyranoside) in the bacterial host BL21(DE3). This polypeptide was used to raise an antiserum as described in the next section, and below in 'Antibodies and immunological methods'.

Expression and purification of recombinant proteins from bacteria

PA-cyclin A was expressed in *Escherichia coli* and purified by NTA-Ni^{2+} 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 $CaCl_2$ 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 $CaCl_2$, 0.1 mM $MgSO_4$, 0.25 mM $NaHCO_3$. 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% CO_2 until the cultures reached about 50% confluence. The medium was then changed to DMEM with 0.2% (v/v) foetal calf serum for 48 hours. Cells were released from the G_0 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 Na_2HPO_4, 2 mM KH_2PO_4) and scraped off the plates. 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 PMSF, 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-glycerophosphate, 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-^{32}P]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-^{32}P]ATP, and then the same procedure as described above was followed.

GST-cdk2 kinase assay

Immunoprecipitates of $p40^{MO15}$ 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-^{32}P]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-^{32}P]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.

Phosphoamino-acid analysis

One-dimensional phosphoamino-acid analysis after partial acid hydrolysis was performed on $^{32}PO_4$-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.

Antibodies and immunological methods

An anti-mouse p40^{MO15} 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 recognise mouse cyclin A and cyclin B1, respectively. Rabbit anti-*Xenopus* p40^{MO15} antiserum was raised against a synthetic peptide corresponding to the C-terminal 11 amino acids of *Xenopus* p40^{MO15} (CADQKDIAKKLSF). Rabbit anti-GST antiserum was as described by Poon et al. (1993). Rabbit anti-human p33^{cdk2} antiserum was raised against a synthetic peptide corresponding to the C-terminal 15 amino acid residues of human p33^{cdk2} (PFFQDVTKPVPHLRL); this antiserum also recognises mouse p33^{cdk2}. Rabbit anti-*Xenopus* p33^{cdk2} antiserum was raised against bacterially expressed *Xenopus* p33^{cdk2}. Anti-p34^{cdc2} monoclonal antibody (A17) was raised against the C-terminal half of *Xenopus* p34^{cdc2} as described by Kobayashi et al. (1991); this antibody also recognises mouse and human p34^{cdc2}. Immunoblotting and immunoprecipitation were performed as described previously (Poon et al., 1993).

RESULTS

Cell cycle variation of p40^{MO15} level and activity

To study the level and activity of p40^{MO15} 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 $CaCl_2$. 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 p40^{MO15} in the cell extracts was measured by immunoblotting with anti-p40^{MO15} 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 p40^{MO15} was measured by the ability of p40^{MO15} immunoprecipitates to phosphorylate GST-cdk2 (bottom panel). Fig. 1 shows that both the level of p40^{MO15} and its activity was the same in M-phase and interphase extracts.

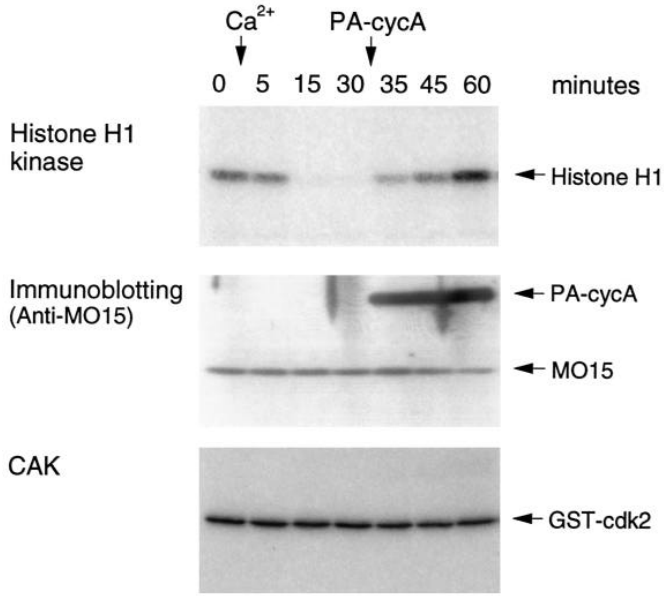

Fig. 1. Cell cycle variation of p40^{MO15} activity in *Xenopus* eggs. *Xenopus* CSF-arrested egg extract was made 0.4 mM in $CaCl_2$ and 100 μg/ml cycloheximide and incubated at 23°C. After 30 minutes, 100 μg/ml of PA-cyclin A was added. Samples were taken at the indicated time points and frozen in liquid N_2. Each sample was divided into three portions, which were assayed for histone H1 kinase (top panel), immunoblotted with anti-p40^{MO15} antibody (middle panel), and immunoprecipitated with anti-p40^{MO15} antibody followed by GST-cdk2 kinase assay (lower panel).

Xenopus somatic cultured cells (WAK cells) can be blocked at the G_1/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 p40^{MO15} was found in WAK cells after release from the aphidicolin block (data not shown).

To study p40^{MO15} activity in somatic cell cycles in more detail, we raised an antiserum against the bacterially expressed C-terminal half of mouse p40^{MO15} (Stepanova et al., 1994). The anti-mouse p40^{MO15} antiserum recognised a 40 kDa protein in immunoblots of Swiss 3T3K cell extracts, but did not recognise the p40^{MO15} 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 p40^{MO15} 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 (G_0), and can be released from G_0 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 G_0 rather than G_1-phase. Almost

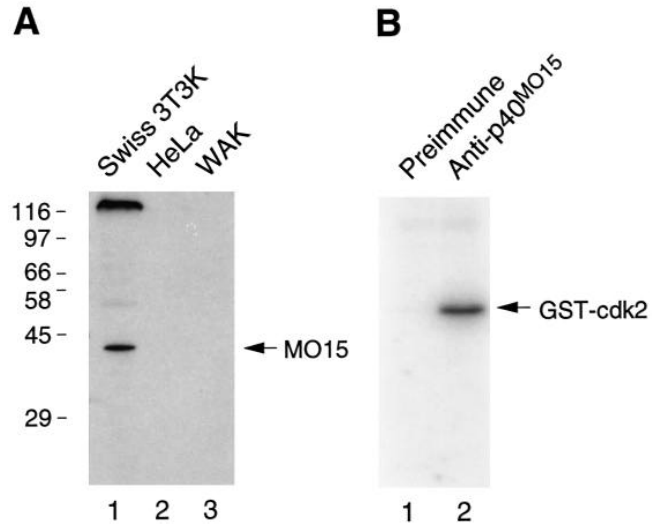

Fig. 2. Anti-mouse $p40^{MO15}$ antibody recognises $p40^{MO15}$ by immunoblotting and immunoprecipitation. (A) Equal amounts (15 μg) of cell extracts made from Swiss mouse 3T3K cells (lane 1), human HeLa cells (lane 2) and *Xenopus* WAK cells were immunoblotted with anti-mouse $p40^{MO15}$ antibody, and the immunoblot was developed with ECL system. (B) Swiss mouse 3T3K cell extract was immunoprecipitated with preimmune serum (lane 1) or anti-mouse $p40^{MO15}$ serum (lane 2). The GST-cdk2 kinase activity associated with the immunoprecipitates was assayed as described in Materials and Methods. The phosphorylated gel band of GST-cdk2 was detected with a phosphorimager (Molecular Dynamics).

all the cells were in S-phase 18 hours after serum release; they reached G_2-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 G_0-arrested cells was 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 G_1-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 G_0 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 reduced to about 25% of serum-replete cultures, whereas $p34^{cdc2}$ was undetectable in G_0 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 serum stimulation, rising to a peak at 21 hours in late G_2- 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. 3J top panel shows the quantitation 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 unfertilised 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 interphase 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 chromatography 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.

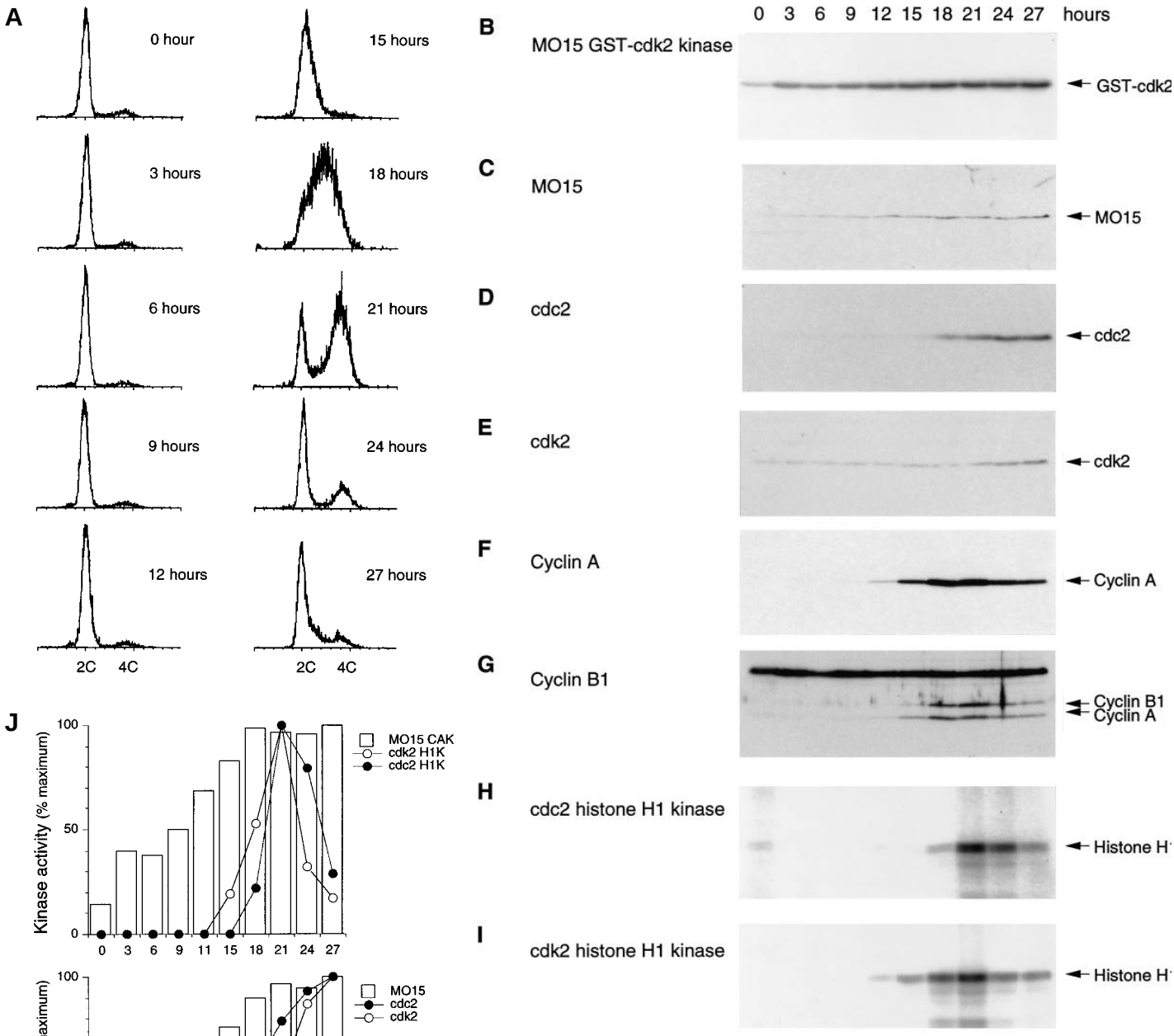

Fig. 3. p40^{MO15} in synchronised Swiss 3T3K cells. (A) Swiss 3T3K cells were synchronised by serum starvation as described in the text. About 5×10^6 cells were taken at the indicated time points, fixed in 70% ethanol and stained with propidium iodide prior to FACS analysis. The vertical axis indicates 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-p40^{MO15} antibody and the GST-cdk2 kinase activity was assayed. The cell extracts were dissolved in SDS-sample buffer and immunoblotted with (C) anti-p40^{MO15} antibody; (D) anti-p34^{cdc2} antibody (A17); (E) anti-p33^{cdk2} 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) p34^{cdc2} and (I) p33^{cdk2} immunoprecipitates were assayed as described in Materials and Methods. (J) Quantitation of the GST-cdk2 kinase activity associated with p40^{MO15} and the histone H1 kinase activity associated with p34^{cdc2} and p33^{cdk2} (top panel), the amount of p40^{MO15}, p34^{cdc2}, p33^{cdk2} (middle panel), cyclin A and cyclin B1 (bottom panel).

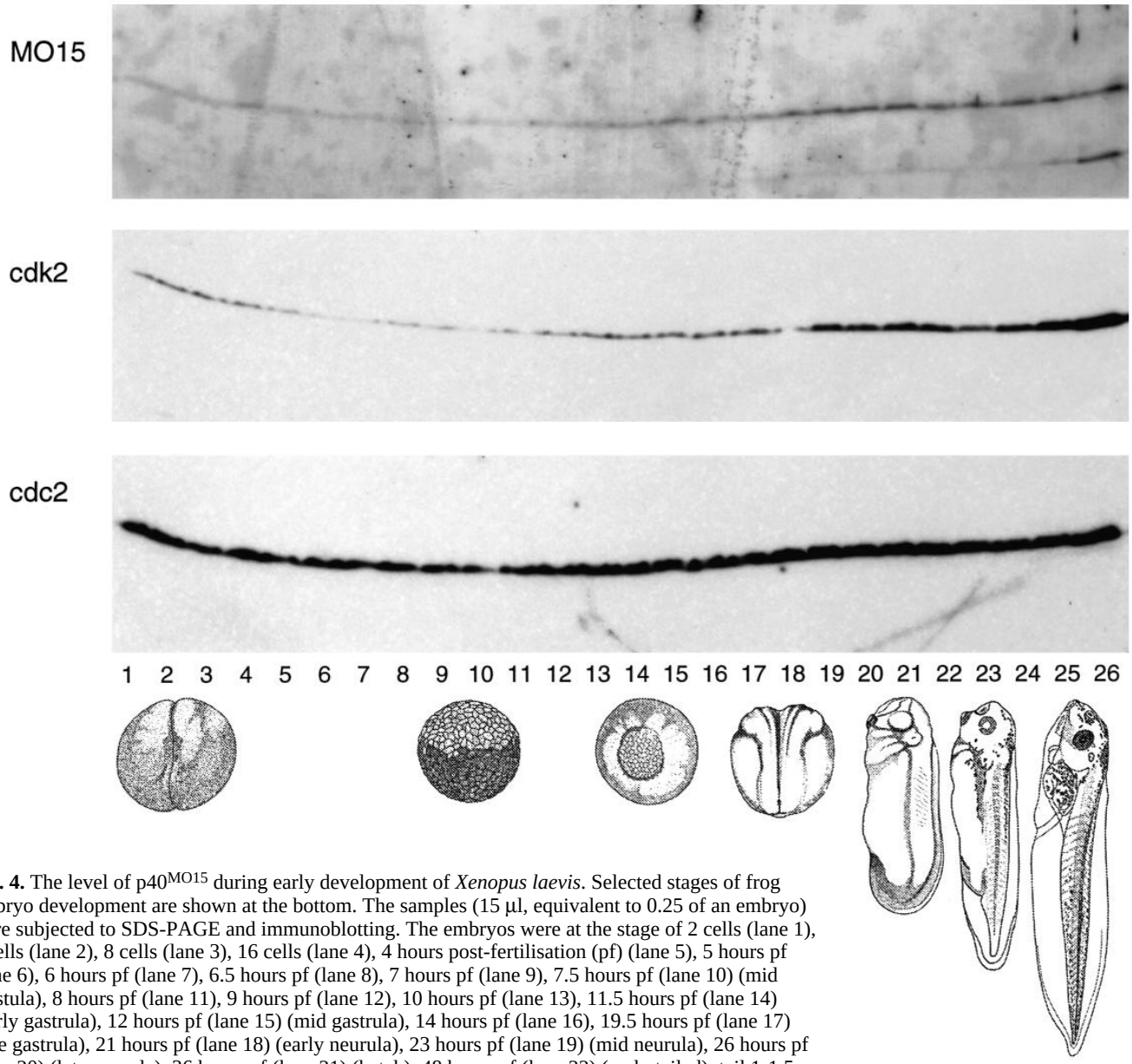

Fig. 4. The level of p40^{MO15} during early development of *Xenopus laevis*. Selected stages of frog embryo development are shown at the bottom. The samples (15 µl, equivalent to 0.25 of an embryo) were subjected to SDS-PAGE and immunoblotting. The embryos were at the stage of 2 cells (lane 1), 4 cells (lane 2), 8 cells (lane 3), 16 cells (lane 4), 4 hours post-fertilisation (pf) (lane 5), 5 hours pf (lane 6), 6 hours pf (lane 7), 6.5 hours pf (lane 8), 7 hours pf (lane 9), 7.5 hours pf (lane 10) (mid blastula), 8 hours pf (lane 11), 9 hours pf (lane 12), 10 hours pf (lane 13), 11.5 hours pf (lane 14) (early gastrula), 12 hours pf (lane 15) (mid gastrula), 14 hours pf (lane 16), 19.5 hours pf (lane 17) (late gastrula), 21 hours pf (lane 18) (early neurula), 23 hours pf (lane 19) (mid neurula), 26 hours pf (lane 20) (late neurula), 36 hours pf (lane 21) (hatch), 48 hours pf (lane 22) (early tailed), tail 1-1.5 mm (lane 23), tail <5 mm (lane 24), tail >5 mm (lane 25), tail 1 cm (lane 26). The samples were immunoblotted with anti-p40^{MO15} antibody (top panel), affinity-purified anti-p33^{cdk2} antiserum (middle panel), or anti-p34^{cdc2} antibody A17 (bottom panel).

Phosphorylation of p40^{MO15} by cell extracts

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 [γ-^{32}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^{MO15}, GST-MO15 was incubated with *Xenopus* CSF-arrested egg extract, interphase egg extract

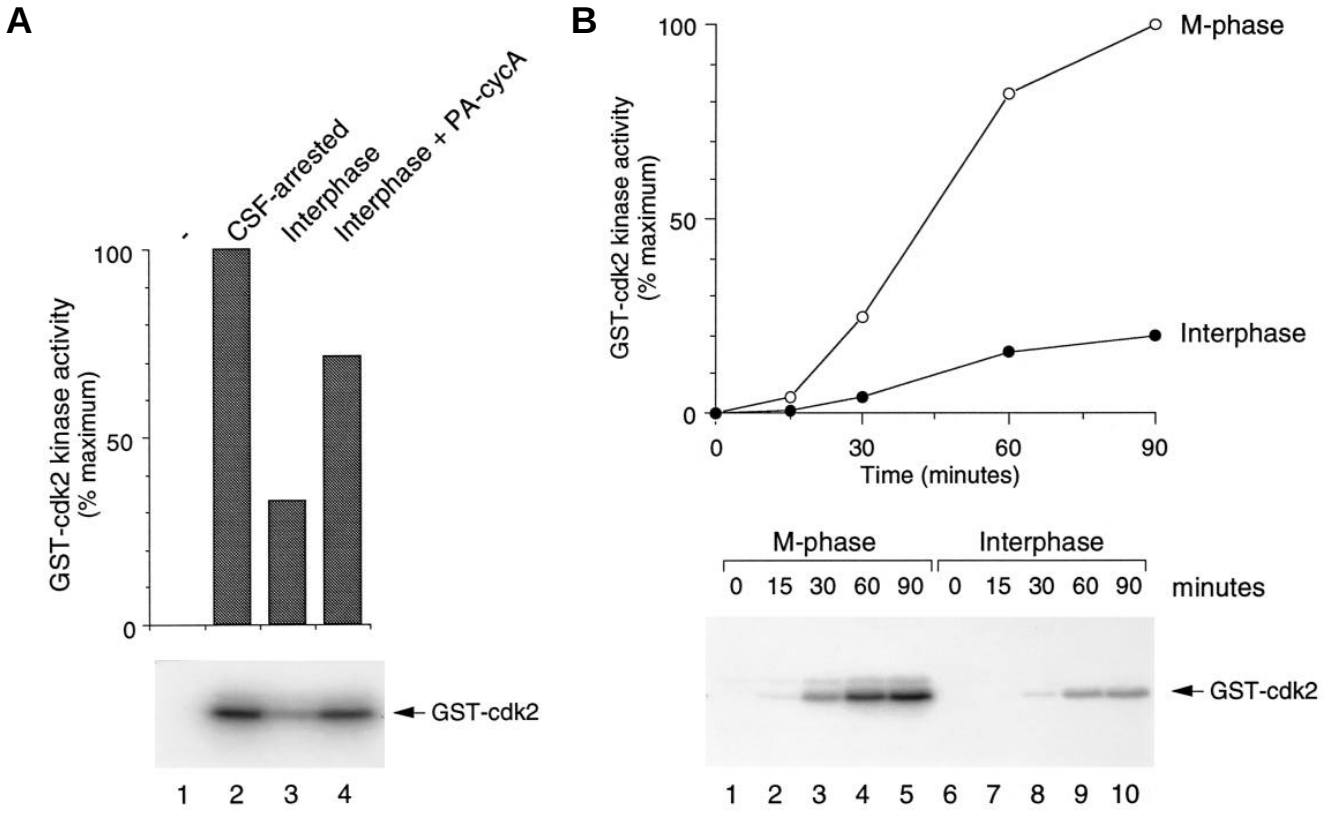

Fig. 5. Activation of GST-MO15 by cell extracts at M-phase and interphase. (A) Buffer (lane 1), *Xenopus* CSF-arrested egg extract (lane 2), interphase egg extract (lane 3), or interphase egg extract treated with PA-cyclin A protein (lane 4) were incubated with GST-MO15 as described in Materials and Methods. GST-MO15 was then harvested with GSH-Sepharose and assayed for its GST-cdk2 kinase activity (lower panel). The upper panel shows the quantitation of the lower panel. (B) CSF-arrested egg extract (lanes 1-5) or interphase egg extract (lanes 6-10) were incubated with GST-MO15 as described in Materials and Methods. GST-MO15 was harvested at the indicated time points and assayed for its GST-cdk2 kinase activity (lower panel). The upper panel shows the quantitation of the lower panel.

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 $p34^{cdc2}/p33^{cdk2}$ was activated (in CSF-arrested extract and PA-cyclin A-treated extract), it was possible that GST-MO15 was directly phosphorylated by $p34^{cdc2}/p33^{cdk2}$. The cyclin-dependent kinases $p34^{cdc2}$ and $p33^{cdk2}$ have a relatively rigid phosphorylation site specificity of a serine or threonine residue followed by a proline. *Xenopus* $p40^{MO15}$ contains two putative serine phosphorylation sites for $p34^{cdc2}/p33^{cdk2}$ at S170 and S186. Among $p40^{MO15}$ 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 $p33^{cdk2}$) 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 could be phosphorylated by cyclin B:$p34^{cdc2}$-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

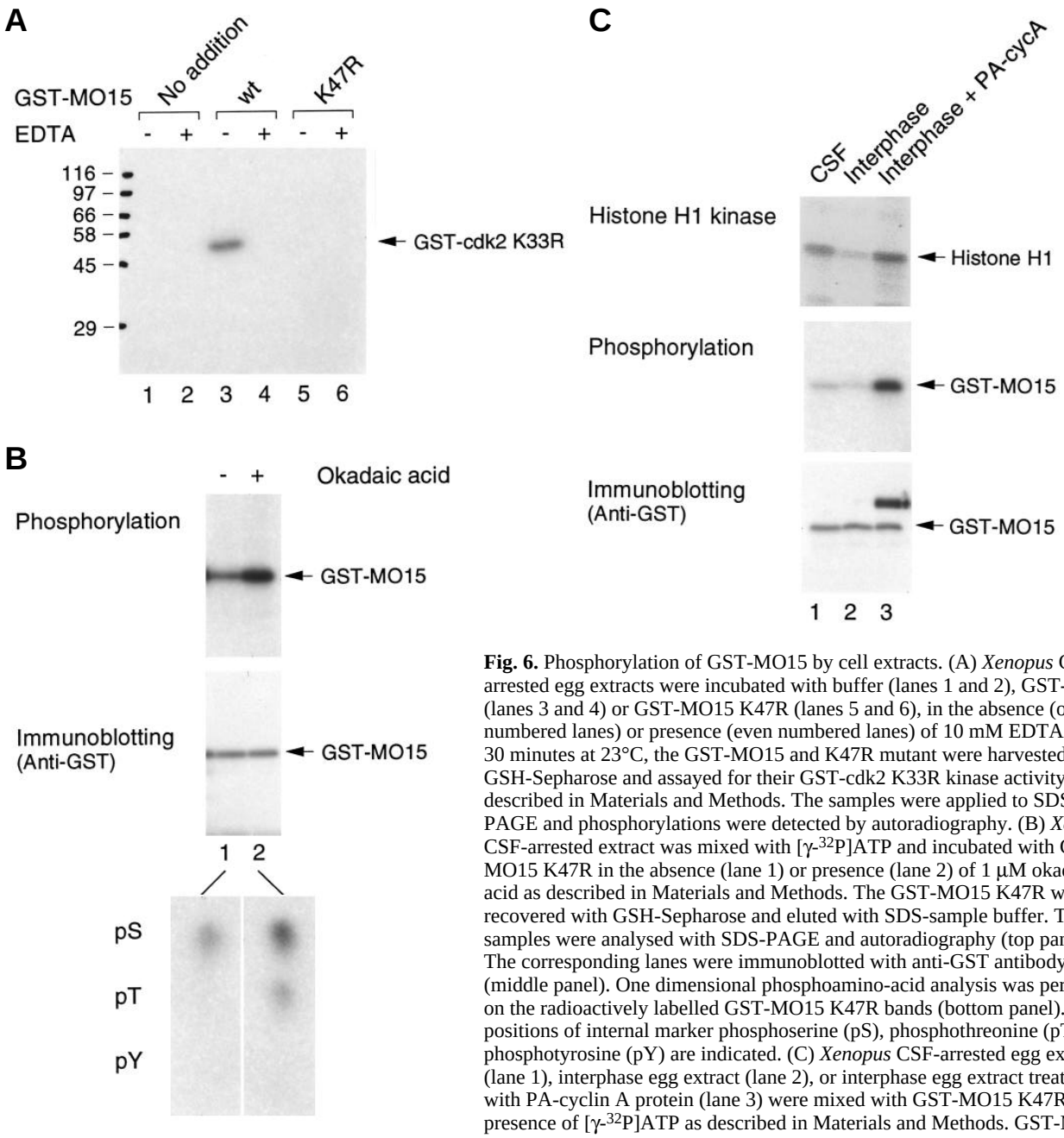

Fig. 6. Phosphorylation of GST-MO15 by cell extracts. (A) *Xenopus* CSF-arrested egg extracts were incubated with buffer (lanes 1 and 2), GST-MO15 (lanes 3 and 4) or GST-MO15 K47R (lanes 5 and 6), in the absence (odd numbered lanes) or presence (even numbered lanes) of 10 mM EDTA. After 30 minutes at 23°C, the GST-MO15 and K47R mutant were harvested with GSH-Sepharose and assayed for their GST-cdk2 K33R kinase activity as described in Materials and Methods. The samples were applied to SDS-PAGE and phosphorylations were detected by autoradiography. (B) *Xenopus* CSF-arrested extract was mixed with [γ-^{32}P]ATP and incubated with GST-MO15 K47R in the absence (lane 1) or presence (lane 2) of 1 μM okadaic acid as described in Materials and Methods. The GST-MO15 K47R was then recovered with GSH-Sepharose and eluted with SDS-sample buffer. The samples were analysed with SDS-PAGE and autoradiography (top panel). The corresponding lanes were immunoblotted with anti-GST antibody (middle panel). One dimensional phosphoamino-acid analysis was performed on the radioactively labelled GST-MO15 K47R bands (bottom panel). The positions of internal marker phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) are indicated. (C) *Xenopus* CSF-arrested egg extract (lane 1), interphase egg extract (lane 2), or interphase egg extract treated with PA-cyclin A protein (lane 3) were mixed with GST-MO15 K47R in the presence of [γ-^{32}P]ATP as described in Materials and Methods. GST-MO15 K47R was then recovered with GSH-Sepharose and phosphorylation was detected with SDS-PAGE and autoradiography (middle panel). The corresponding samples were immunoblotted with anti-GST antibody to detect the harvested GST-MO15 K47R (bottom panel). The total histone H1 kinase activities of the egg extracts were assayed and subjected to SDS-PAGE and autoradiography (top panel).

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 $p33^{cdk2}$, 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 $p34^{cdc2}$/$p33^{cdk2}$ 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 $p40^{MO15}$ activity

We show in this paper that there is no substantial variation on the level or kinase activity of $p40^{MO15}$ (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 G_0 state by serum deprivation do the level and the kinase activity of $p40^{MO15}$

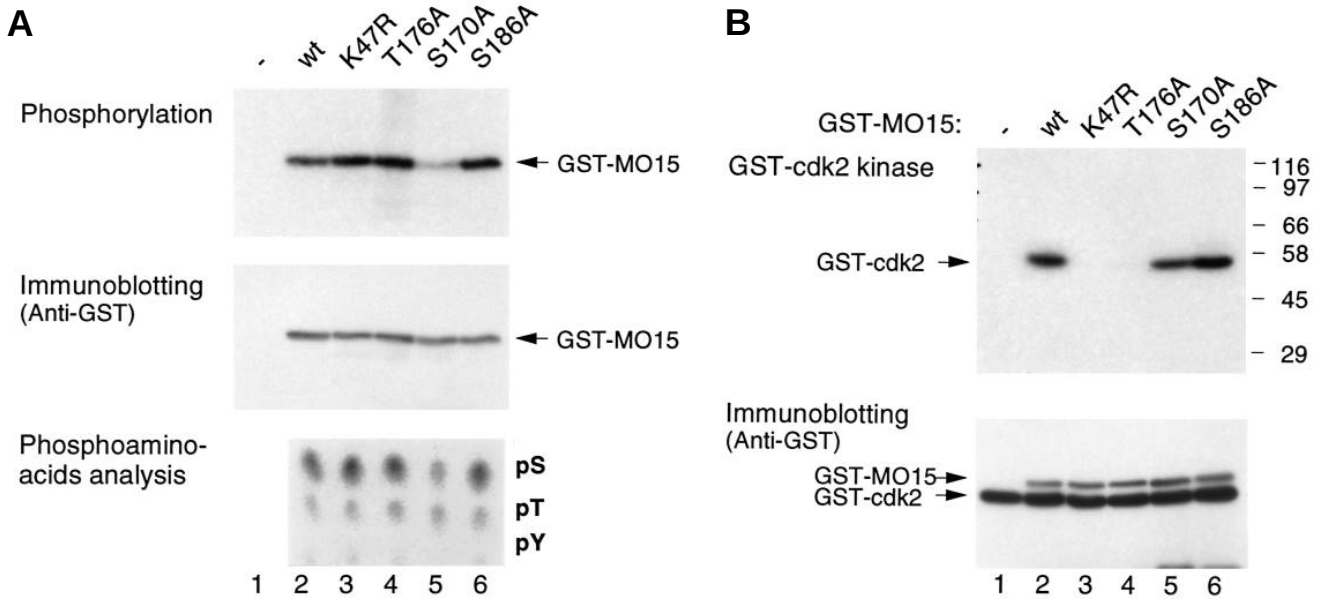

Fig. 7. Phosphorylation and activation of GST-MO15 mutants. (A) *Xenopus* CSF-arrested extract was mixed with [γ-^{32}P]ATP and incubated with buffer (lane 1), 100 μg/ml of GST-MO15 (lane 2), K47R mutant (lane 3), T176A mutant (lane 4), S170A mutant (lane 5) or S186A mutant (lane 6) as described in Materials and Methods. The GST-MO15 or mutants were then recovered with GSH-Sepharose and dissolved in SDS-sample buffer. The samples were analysed with SDS-PAGE and autoradiography (top panel). The corresponding samples were immunoblotted with anti-GST antibody (middle panel). The corresponding radioactively labelled GST-MO15 or mutants bands were subjected to one-dimensional phosphoamino-acid analysis (bottom panel). The positions of internal marker phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) are indicated. (B) *Xenopus* CSF-arrested egg extract was incubated with buffer (lane 1), 100 μg/ml of GST-MO15 (lane 2), GST-MO15 K47R mutant (lane 3), T176A mutant (lane 4), S170A mutant (lane 5) or S186A mutant (lane 6). After 60 minutes at 23°C, the GST-MO15 and mutants were harvested with GSH-Sepharose and assayed for their kinase activity towards GST-cdk2 K33R as described in Materials and Methods (upper panel). The corresponding samples were immunoblotted with anti-GST antibody (lower panel).

become diminished. It is unlikely that the decrease in activity of p40^{MO15} in G_0 is important in reducing the activity of either p34^{cdc2} or p33^{cdk2}, 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 p34^{cdc2} and p33^{cdk2} in G_0 can readily be ascribed to the combined lack of p34^{cdc2}, p33^{cdk2} and cyclins, rather than of p40^{MO15}. These results do not exclude the possibility that p40^{MO15} might be regulated in the cell cycle by other means, such as subcellular compartmentation. It is also possible that the p40^{MO15} 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 p40^{MO15} towards substrates other than p34^{cdc2}/p33^{cdk2} may vary in the cell cycle, although at present, no such substrates have been identified.

Phosphorylation regulation of p40^{MO15}

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 p34^{cdc2}, phosphorylation of T176 of p40^{MO15} 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 T176 is only a minor phosphorylation site under these conditions, though the lack of activity of the 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 may be important in the regulation of p40^{MO15} activity because it can be phosphorylated by active p34^{cdc2}/p33^{cdk2} in vitro (unpublished data), which are themselves activated by p40$^{MO15.}$ Hence, there could be some kind of feedback control. Furthermore, because S170 is close to the putative activating phosphorylation site of p40^{MO15} (T176), phosphorylation of S170 may affect the conformation of T176. Although the S170A mutation does not affect the activation of

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 there are more putative partner subunits available for GST-MO15 in M-phase extracts than in interphase extracts, or that their rates of exchange differ in M-phase and 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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