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REGULATION BY PROTEIN PHOSPHATASE 2C IN *XENOPUS* OOCYTES*

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Fully grown *Xenopus* oocyte is arrested at prophase I of meiosis. Re-entry into meiosis depends on the activation of MPF (M-phase promoting factor or cyclin B·Cdc2 complex), triggered by progesterone. The prophase-arrested oocyte contains a store of Cdc2. Most of the protein is present as a monomer whereas a minor fraction, called pre-MPF, is found to be associated with cyclin B. Activation of Cdc2 depends on two key events: cyclin binding and an activating phosphorylation on Thr-161 residue located in the T-loop. To get new insights into the regulation of Thr-161 phosphorylation of Cdc2, monomeric Cdc2 was isolated from prophase oocytes. Based on its activation upon cyclin addition and detection by an antibody directed specifically against Cdc2 phosphorylated on Thr-161, we show for the first time that the prophase oocyte contains a significant amount of monomeric Cdc2 phosphorylated on Thr-161. PP2C, a Mg^{2+}-dependent phosphatase, negatively controls Thr-161 phosphorylation of Cdc2. The unexpected presence of a population of free Cdc2 already phosphorylated on Thr-161 could contribute to the generation of the Cdc2 kinase activity threshold required to initiate MPF amplification.

The fully grown *Xenopus* oocyte is physiologically arrested at the diplotene stage of meiotic prophase; it contains a maternal store of Cdc2 or Cdk1 (cyclin-dependent kinase). The majority of the protein is present as a monomer in the cytoplasmic compartment of the oocyte, whereas a minor fraction (10% as estimated by Western blotting) is found to be associated with B2 and B5 cyclins (1, 2). The cyclin B·Cdc2 complex, which accumulates during oogenesis, is maintained inactive by two inhibitory phosphorylations on Thr-14 and Tyr-15 of Cdc2 catalyzed by the membrane-associated Myt1 kinase (3, 4). Another phosphorylation of Cdc2, on the Thr-161 residue located in the T-loop of the protein, is known to be required for Cdc2 kinase activation (5). The inactive cyclin B·Cdc2 complex present in fully grown oocyte, also known as pre-MPF,[1] contains a triphosphorylated Cdc2 subunit on Thr-14, Tyr-15, and Thr-161 (6). Two hypotheses can be envisaged concerning the timing and the location of Cdc2 phosphorylation on Thr-161 and the enzymes responsible on this process during oogenesis: 1) Newly synthesized cyclin B associates with Cdc2 in the cytoplasm. Then the neocomplex is translocated to the nucleus where it becomes a substrate of a CDK-activating kinase (CAK), composed of CDK7, cyclin H, and the assembly factor MAT1, a complex known to be strictly located within the *Xenopus* oocyte nucleus (7, 8). CAK exhibits a stronger affinity for cyclin-associated CDKs than for monomeric CDKs (9). To prevent premature activation of Cdc2, the complex needs to be inactivated by phosphorylations on Thr-14 and Tyr-15 of Cdc2 by the ER membrane-associated Myt1 kinase (3), to accumulate as pre-MPF in the cytoplasm. 2) Cyclin-free Cdc2 is a substrate of another cytoplasmic CAK. In *Saccharomyces cerevisiae*, the only known CAK is a cytoplasmic monomeric enzyme called Cak1 or Civ1 (10–12). In contrast to the CDK7·cyclin H complex, it preferentially phosphorylates monomeric CDKs rather than cyclin-associated CDKs (9). Recently, a "monomeric CAK" activity has been also detected in human cells (13, 14). If such an enzyme is expressed, then monomeric Cdc2 would be phosphorylated on Thr-161 in the cytoplasm prior its association with newly synthesized cyclin B, and, in the case of the growing oocyte, prior to its inactivation by the membrane-associated Myt1 kinase, leading to pre-MPF formation.

During progesterone-induced meiotic maturation, the abrupt activation of pre-MPF into MPF occurs through an autoamplification process whereby the protein phosphatase Cdc25 removes the inhibitory phosphates on Thr-14 and Tyr-15 of Cdc2 (6). A two-step mechanism, involving proteins such as protein phosphatase 2A and Plx1 kinase, allows active Cdc2 to positively regulate Cdc25 (15). A major unanswered question is how the feedback loop between Cdc25 and Cdc2 is initiated. One possibility could be that an unstable or a neosynthetized "Cdc25-like" phosphatase, such as Cdc25B, is activated before Cdc25C and serves as a threshold for the Cdc2-Cdc25C autoamplification loop. Until now, no experimental evidence supports this hypothesis in the *Xenopus* oocytes undergoing meiotic division. Another possibility is that, upon progesterone stimulation, a newly synthesized cyclin, or another Cdc2 partner, would associate with free Cdc2; the neoformed complex would then escape inactivating phosphorylations by Myt1 kinase and would serve as a threshold to initiate MPF autoamplification (15–17). In this context, the presence of monomeric

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ438209.

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[1] The abbreviations used are: MPF, M-phase promoting factor; CDK, cyclin-dependent kinase; CAK, CDK-activating kinase; Cak1, *S. cerevisiae* CDK-activating kinase 1; GVBD, germinal vesicle breakdown; PP2C, protein phosphatase 2C; DTT, dithiothreitol; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; GSH, reduced glutathione; PKA, protein kinase A; OA, okadaic acid; KAP, CDK-associated phosphatase.

Cdc2 already phosphorylated on Thr-161 would favor the formation of this small starter amount of active MPF.

The phosphorylation of Cdc2 on Thr-161 represents a potential key regulation step at two essential periods of the oocyte development; first, during late oogenesis when pre-MPF accumulates in prophase-arrested oocyte, and second, at the time preceding GVBD, when pre-MPF is activated. A major insight is to identify the enzymes, kinase and phosphatase, that control this critical event.

In a first approach to understand how the Thr-161 phosphorylation of Cdc2 is regulated, we decided to undertake a biochemical purification of cyclin B-free Cdc2 and analyzed its Thr-161 phosphorylation level. The major observation of this study indicates that fully grown resting oocyte contains a significant amount of monomeric Cdc2 phosphorylated on Thr-161, whose phosphorylation level is negatively regulated by a Mg^{2+}-dependent phosphatase, PP2C.

EXPERIMENTAL PROCEDURES

Materials

Xenopus laevis adult females (CNRS, Rennes, France) were bred and maintained under laboratory conditions. [γ-^{32}P]ATP (6000 Ci/mmol, NEG502Z) was purchased from PerkinElmer Life Sciences. Reagents, unless otherwise specified, were from Sigma Chemical Co.

Xenopus Oocyte Extracts

Fully grown *Xenopus* prophase oocytes were obtained as described previously (18). For extract preparations, oocytes were lysed in 4 volumes of EB (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, pH 7.3) or modified EB (80 mM β-glycerophosphate, 10 mM EDTA, 30 mM NaCl, 1 mM DTT, pH 7.3) supplemented with 10 mM ATP, 50 mM NaF, 100 μM sodium orthovanadate, and protease inhibitor mixture (Sigma P8340). Lysates were centrifuged at 100,000 $\times$ *g*, and the supernatants were recovered and termed "cytosolic extracts" or "S100." Proteins of cytosolic extracts were precipitated by salting out using ammonium sulfate, successively 40 and 60%, as described in a previous study (15). Ammonium sulfate pellets, respectively, P40 and P60, were stored at −80 °C for further analysis.

Gel Filtration

P40 and P60 precipitates from 200 oocytes were resuspended in 160 μl of column buffer (EB or modified EB, adjusted to 0.1 M NaCl) and then chromatographed on a Superose 12 gel filtration column (Amersham Biosciences) at 0.5 ml/min. Ten fractions of 1 ml were collected and subject to Western blot and kinase and phosphatase assays.

Immunoblotting

Proteins were separated on 12% SDS-PAGE (Amresco) and transferred to nitrocellulose filters (Schleicher and Schuell). Anti-*Xenopus* cyclin B2 and cyclin B1 antibodies were obtained from goats immunized with inclusion bodies containing bacterially expressed *Xenopus* cyclins B2 and B1 and affinity-purified. The monoclonal mouse anti-*Xenopus* Cdc2 antibodies (mixture of A17 and 3E1) were initially described in a previous study (19). The anti-MO15 and anti-Cak1 polyclonal rabbit antibodies were described previously (Refs. 8 and 10, respectively). Polyclonal rabbit anti-phospho-Cdc2 (Tyr-15) and anti-phospho-Cdc2 (Thr-161) antibodies were purchased from Cell Signaling Technology, and polyclonal rabbit anti-PSTAIR antibody and polyclonal sheep anti-human PP2Cα were purchased from Upstate Biotechnology. The primary antibodies were detected with appropriated horseradish peroxidase-conjugated second antibodies (Jackson ImmunoResearch laboratories) and the Western blot Chemiluminescence Renaissance kit from PerkinElmer Life Sciences.

Cdc2 Activation and Cdk2 Phosphorylation

Recombinant GST-Cdc2—GSH-Sepharose beads bound to purified and refolded GST-Cdc2 were washed in kinase buffer (50 mM Tris-HCl, pH 7.2, 1 mM DTT, 15 mM $MgCl_2$, 5 mM EGTA) and then incubated for 30 min at 30 °C in kinase buffer in the presence of 100 μM ATP and various effectors: Cak1 (0.06 μg/μl), GST-cyclin A (0.1–0.2 μg/μl) or His-cyclin B1 (0.1 μg/μl). For histone H1 kinase assay, 0.2 mg/ml histone H1 (Roche Diagnostics) and 1 μCi of [γ-^{32}P]ATP were added for a further 15 or 30 min at 30 °C. The reaction was stopped by adding Laemmli buffer (20) and by boiling for 3 min.

Endogenous Monomeric Cdc2—Ammonium sulfate was removed from P40 and P60 by ultrafiltration with an Ultrafree Biomax system (Millipore). The amount of proteins recovered in each fraction was evaluated by Bradford analysis (21). One oocyte corresponds to 24, 8, 12, and 1.2 μg of proteins, respectively, in S100, P40, P60, and F9. Activation of endogenous Cdc2 present in P60 or F9 was performed under the same conditions as for recombinant Cdc2, by adding Cak1 (0.06 μg/μl), GST-cyclin A (0.2 μg/μl), or His-cyclin B1 (0.1 μg/μl). In some experiments, GST·cyclin A·Cdc2 complexes were recovered by GST-cyclin A binding for 4 h at 4 °C on GSH-agarose beads. After several washes in EB (modified or not) or in kinase buffer, the bead pellets were, respectively, submitted to Western blot analysis or histone H1 kinase assay.

Recombinant GST-Cdk2—Phosphorylation of GST-Cdk2 was performed by incubating the protein (0.1 μg/μl) for 1 h at 30 °C in the presence of Cak1 (0.06 μg/μl) in kinase buffer containing 10 μM ATP and 1 μCi of [γ-^{32}P]ATP. GST-Cdk2 was then ultrafiltrated on a Microcon system (Millipore) to eliminate free [γ-^{32}P]ATP or purified on GSH-agarose beads for 4 h at 4 °C. In some experiments, incubation was performed in the presence of various concentrations of P60 and F9. After GST pull-down, pellets were washed, resuspended in sample buffer, and heated at 100 °C for 3 min, and proteins were separated on 12% SDS-PAGE. The radioactivity incorporated in GST-Cdk2 was revealed by autoradiography and counted after excision from the gel in a Wallac counter.

Phosphatase Assay

Substrate Preparation for PP2C Isolation Assay—Casein (Sigma C4765, 5 mg) was phosphorylated by 250 milliunits of the catalytic subunit of PKA (Sigma P2645) for 2 h at 30 °C in the presence of 100 μM ATP and 250 μCi of [γ-^{32}P]ATP. GST-Cdk2 (1 mg) was phosphorylated by Cak1 (15 μg) for 16 h at 30 °C in the presence of 200 μM ATP and 500 μCi of [γ-^{32}P]ATP. Reactions were stopped by addition of 10 mM EDTA, 30 mM NaF, and 2 mM pyrophosphate. Proteins were then precipitated twice at 0 °C with an equal volume of 90% saturated ammonium sulfate solution. Free nucleotides were removed by chromatography on Sephadex G-25 (Amersham Biosciences).

Phosphatase Reaction—^{32}P-Phosphorylated GST-Cdk2 or ^{32}P-phosphorylated casein was incubated for 20 min at 30 °C in the presence of either F9 (1 μg of proteins), recombinant *Xenopus* PP2C, or fractions from the purification procedure in the presence of bovine serum albumin (5 μg) and various amounts of Mg^{2+} and OA. Reactions were stopped by addition of 10 volumes of 20% trichloroacetic acid, centrifuged for 5 min, and the released ^{32}P label was counted.

Xenopus PP2C Purification

Phosphatase activity was determined using casein phosphorylated by PKA and Cdk2 phosphorylated by Cak1 as substrates. The selected fractions contain a phosphatase activity toward both substrates that is dependent on Mg^{2+} and insensitive to 1 μM okadaic acid. The entire purification procedure was carried out at 4 °C. Ovaries from 30 females were homogenized in 3 volumes of the following buffer: 50 mM Tris-HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, 0.1% β-mercaptoethanol, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride (Pentapharm), 1 mM benzamidine. The lysate was centrifuged at 10,000 $\times$ *g* for 20 min, and the supernatant was filtered through glass wool and centrifuged again at 21,000 $\times$ *g* for 3 h, leading to a cytosolic extract. P40 and P60 were then prepared (15). 30–40% of PP2C was recovered in the P40 while 60–70% was recovered in P60, as estimated by Western blot signal. The P60 fraction was resuspended in 1 liter of buffer A (25 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 0.1% β-mercaptoethanol) and mixed with 500 ml of DEAE-Sepharose Fast-Flow resin equilibrated in buffer A. Fractions were eluted by steps in buffer A containing increasing NaCl concentrations (100 mM, 250 mM, 500 mM, 750 mM, and 1 M). The active fraction, eluted in 500 mM NaCl, was concentrated on Centricon Plus-80 (Amicon) and loaded on a Sephacryl S200 column equilibrated in buffer A plus 150 mM NaCl. The active fraction was desalted on Centricon Plus-80 and then loaded on a UnoQ column (Bio-Rad) equilibrated in buffer A. Proteins were eluted with a linear gradient from 0 to 1 M NaCl in buffer A. PP2C activity was eluted around 500 mM NaCl. NaCl was increased up to 3 M in the active fraction, which was then loaded on a phenyl-Superose HR5/5 column (Amersham Biosciences) equilibrated in buffer A plus 3 M NaCl. Proteins were eluted with a linear gradient from 3 to 0 M NaCl in buffer A, and the phosphatase activity was recovered at around 750 mM NaCl. The active fraction was desalted on Centricon Plus-80 with buffer A, then supplemented with 25 mM $MgCl_2$ and loaded on a 5-ml Hi-Trap

Blue column (Amersham Biosciences) equilibrated with buffer A plus 25 mM $MgCl_2$. Proteins were eluted with a linear gradient from 25 to 0 mM $MgCl_2$ in buffer A, and the active fraction was recovered at 13 mM $MgCl_2$. After concentration by dialysis against 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1% β-mercaptoethanol, and 20% polyethylene glycol 40,000, the active fraction was stored at −80 °C.

Xenopus PP2Cα Cloning

Based on the sequences dd98g09.x1 (December 2000) and dc59g09.y1 (September 2000) of two *X. laevis* expressed sequence tag cDNA clones (IMAGE (National Institutes of Health): 3436624 and 3401440) homologous to the human protein phosphatase 2Cα, two oligoprimers containing *Bgl*II sites (underlined) were designed: 5′-GAA GAT CTC ATG GGA GCA TTT TTA GAT AAG CC-3′ (corresponding to the amino-terminal part of the protein and used as upstream primer), and 5′-GAA GAT CTC TTA CCA CAT ATC ATC TGT TGA TGC-3′ (corresponding to the carboxyl-terminal part of the protein and used as downstream primer). PCR was performed with a mix (50/50) of *Pfu* DNA polymerase and *Taq* DNA polymerase (Promega, #M7741 and #M2661) using a cDNA library from *X. laevis* oocytes (λ ZAP Express phages, kind gift of Dr. J. Maller). The amplified PCR product was subcloned in the pGEM-T easy vector, and the cDNA sequences were determined on automated DNA Sequencer ALF-express (Amersham Biosciences) with a Thermo Sequenase CY5 Dye Terminator kit (Amersham Pharmacia) using T3- and T7-CY5 primers. The entire encoding nucleotide sequence has been deposited at the EMBL nucleotide sequence data base under the accession number AJ438209.

Preparation of Recombinant Proteins

GST- and His-tagged recombinant proteins were expressed and purified as described in a previous study (19), using the following plasmids: human GST-cyclin A (kind gift of Dr. C. Bréchot, INSERM, France), *Xenopus* GST-Cdk2, *Xenopus* wild type GST-Cdc2, and *Xenopus* Thr-161 → Ala mutant GST-Cdc2 (kind gifts of Dr. T. Hunt, Imperial Cancer Research Fund, UK). *S. cerevisiae* His-Cak1 protein and human His-cyclin B1 protein were kindly provided by Dr. C. Mann (Commissariat ă l'Energie Aromique, Saclay, France) and Dr. B. Ducommun (CNRS, Toulouse, France), respectively.

Bacterially produced *Xenopus* GST-Cdc2 is inactive and requires a refolding step (19, 22). This was performed by incubating 1 μg of wild type or Thr-161 → Ala mutant GST-Cdc2 in 2.5 μl of prophase oocyte extracts, prepared as described previously (23), for 30 min at room temperature. After refolding, GST-Cdc2 was isolated using GSH-Sepharose beads (Amersham Biosciences).

Xenopus PP2Cα cDNA was cloned into the expression vector pThio-HisB (Invitrogen). Expression of recombinant ThioHis-PP2C was induced with 0.5 mM isopropylthio-β-D-galactoside. The bacterial pellet was lysed in 20 mM NaH_2PO_4, 500 mM NaCl, *N*-octylglucoside (0.5% v/v), 1% (v/v) protease inhibitor mixture (Sigma P8340) and centrifuged (10 min, 14,000 × *g*, 4 °C). The supernatant was chromatographed on a nickel column (Probond, Invitrogen), and the imidazole eluate was chromatographed on a phenyl arsine oxide-agarose column (Thiobond, Invitrogen). Step-elution was performed with β-mercaptoethanol from 50 mM to 1 M. Fractions of interest were dialyzed and concentrated in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and stored at −80 °C before use.

RESULTS

Partial Purification of Cyclin-free Cdc2—To separate monomeric Cdc2 from cyclin B·Cdc2 complex, prophase oocytes were homogenized in EB, a buffer known to preserve MPF activity (24). Cytosolic extracts (S100) were obtained by 100,000 × *g* centrifugation and were then fractionated by ammonium sulfate precipitation. The 40% and the 60% ammonium sulfate precipitates were termed, respectively, P40 and P60 hereafter and analyzed by Western blotting. Cyclins B2 and B1 were exclusively recovered in P40 (Fig. 1*A*), as well as cyclins B4 and B5 (data not shown). It is well documented that inactive Cdc2 from prophase oocytes migrates as doublet on SDS-PAGE, the *upper band* corresponding to cyclin-associated Cdc2 and the *lower band* to free Cdc2 (15, 25). Indeed, a monoclonal antibody directed against *Xenopus* Cdc2 recognized two bands in P40 (Fig. 1*A*). The *upper band* was recognized by an antibody directed against Tyr-phosphorylated Cdc2 (Fig. 1*A*). The *lower band* migrates as monomeric Cdc2 (see fractionation by gel filtration in Fig. 1*B*). P40 therefore contains a mixed population of monomeric Cdc2 and Tyr-phosphorylated Cdc2 associated with B-cyclins, corresponding to pre-MPF. Supporting this conclusion, addition of recombinant Cdc25 phosphatase to P40 leads to a strong Cdc2 kinase activation (15).

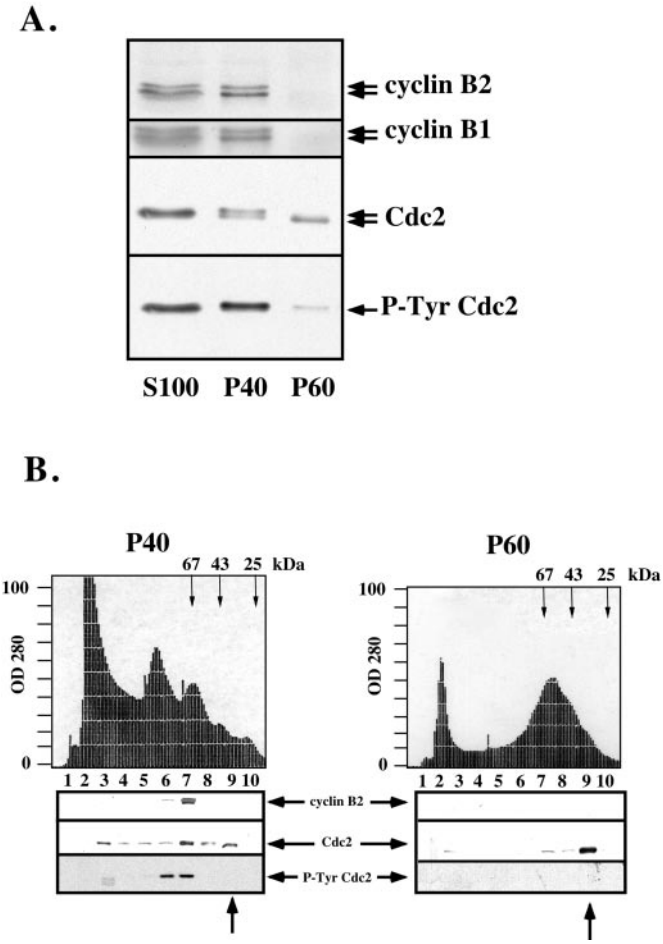

FIG. 1. **Separation of monomeric Cdc2 from cyclin B·Cdc2 heterodimer.** *A*, the 100,000 × *g* (S100) cytosolic extract and the P40 and the P60 ammonium precipitates were analyzed by Western blotting, using (from the *top panel* to the *lower panel*) anti-cyclin B2 antibody, anti-cyclin B1 antibody, anti-Cdc2 antibody, anti-phospho-Tyr-Cdc2 antibody (*P-Tyr Cdc2*). *B*, Superose 12 chromatography of P40 and P60. Optical density (*OD*) was measured at 280 nm in the Superose 12 fractions. Molecular mass markers (Amersham Biosciences) are indicated. The Superose 12 fractions were analyzed by Western blotting with the antibodies against cyclin B2, Cdc2, and phospho-Tyr-Cdc2 (*P-Tyr Cdc2*). The fractions containing monomeric Cdc2 are indicated by the *bottom arrows*.

A similar analysis was conducted in P60. Cyclins B1, B2, B4, and B5 were not detected in this fraction (Fig. 1*A* and data not shown). A single band was detected by the anti-Cdc2 antibody, migrating at the same position as the lower Cdc2 band present in P40 (Fig. 1*A*). In some experiments, a faint signal could be observed with the anti-Tyr-phosphorylated Cdc2 antibody (Fig. 1*A*), probably due to the unspecific recognition of some unphosphorylated Cdc2. Indeed, addition of recombinant Cdc25 phosphatase to P60 did not generate any Cdc2 kinase activation (data not shown), excluding the presence of pre-MPF in this fraction. Therefore, P60 contains exclusively a subpopulation of cyclin B-free Cdc2. It is therefore possible to reproducibly separate cyclin-free from cyclin-bound Cdc2 through a single step of ammonium sulfate fractionation.

P40 and P60 were further fractionated by gel filtration on Superose 12 column, leading to 10 fractions, F1 to F10. After fractionation of P40, Cdc2 was recovered mainly in three frac-

tions, F3, F7, and F9. F7 contains cyclin B2 and Tyr-phosphorylated Cdc2 (Fig. 1*B*), indicating that pre-MPF is segregated in this fraction, an observation in agreement with the expected molecular weights of proteins in this fraction. In contrast, F9 contains cyclin-free Cdc2 molecules that are not phosphorylated on Tyr-15 and that are presumably monomeric (Fig. 1*B*), according to the molecular weight range of proteins recovered in this fraction. Interestingly, cyclin-free Cdc2 was also found in high molecular weight complexes in F3 (Fig. 1*B*).

Cdc2 present in P60 was mainly recovered in a single fraction, F9, corresponding to its molecular mass (34 kDa), whereas B-cyclins could not be detected in any fraction (Fig. 1*B*). No signal could be detected by the anti-phospho Tyr-Cdc2 antibody in F9 (Fig. 1*B*). This result strongly argues that Cdc2 is present as a monomer in both P60 and F9 fractions. Therefore, this two-step procedure allows the reproducible and rapid isolation of partially purified monomeric Cdc2. In the following study, either P60 or F9 originated from Superose 12 chromatography of P60 were used to analyze free Cdc2.

Monomeric Xenopus Cdc2 Is an in Vitro Substrate of Cak1—The *S. cerevisiae* Cak1 monomeric enzyme is a protein kinase able to phosphorylate yeast CDC28 and *in vitro* recombinant human Cdk2 on the activating Thr residue, Thr-169 and Thr-160, respectively, located in their T-loop (9–12, 26). We first ascertained that the *Xenopus* Thr-161 activating residue of the Cdc2 T-loop is *in vitro* phosphorylated by Cak1. We used recombinant wild type *Xenopus* GST-Cdc2 and Thr-161 → Ala (T161A) mutant GST-Cdc2, a protein that cannot be phosphorylated by CAK enzymes. Both proteins were bacterially produced and refolded, as described (19, 22). The GST-tagged Cdc2 proteins were recovered on GSH-Sepharose beads and then incubated in the presence of either GST-cyclin A or His-cyclin B1, in the presence or in the absence of recombinant Cak1 enzyme. The kinase activity of Cdc2 was then assayed by using histone H1 as substrate. As shown in Fig. 2*A*, recombinant wild type *Xenopus* Cdc2 is activated in a Cak1- and cyclin-dependent manner. In contrast, the T161A Cdc2 mutant is not activated by Cak1 and cyclins. This result shows that *Xenopus* Cdc2 is an *in vitro* substrate of Cak1 and that Thr-161-phosphorylated Cdc2 is directly activable by cyclin A or cyclin B1 binding.

We then tested whether endogenous monomeric Cdc2 present in P60 or F9 could be similarly activated by the Cak1 enzyme. We first established by Western blotting that the only CAK activity described in *Xenopus* oocytes up to now, the CDK7(MO15)·cyclin H complex (27), is not present in P60 but entirely recovered in P40 (Fig. 2*B*). P60 was prepared in EB and was supplemented by either GST-cyclin A or His-cyclin B1. Cdc2 kinase activity was then estimated. Fig. 2*C* shows that cyclin addition is not sufficient to significantly activate Cdc2 kinase. Addition of Cak1 together with cyclins in P60 led to the activation of Cdc2 kinase activity (Fig. 2*C*). Similar results were obtained by using F9 (data not shown). Therefore, *Xenopus* monomeric Cdc2 is a substrate of Cak1, and its Thr-161-phosphorylated form is directly activable by cyclin binding.

A Phosphatase Activity Counteracts Cak1 Enzyme in P60—We next measured the level of histone H1 kinase activity generated by fixed amounts of Cak1 and GST-cyclin A in the presence of increasing amounts of P60, corresponding to increasing amounts of endogenous monomeric Cdc2. Unexpectedly, the level of histone H1 kinase activity generated by GST-cyclin A and Cak1 addition sharply decreased when the amount of P60 containing monomeric Cdc2 increased over 25 μg of proteins (Fig. 3*A*). A similar experiment was performed in F9 and gave identical results (data not shown). This result could be explained by the presence of a phosphatase in P60 and

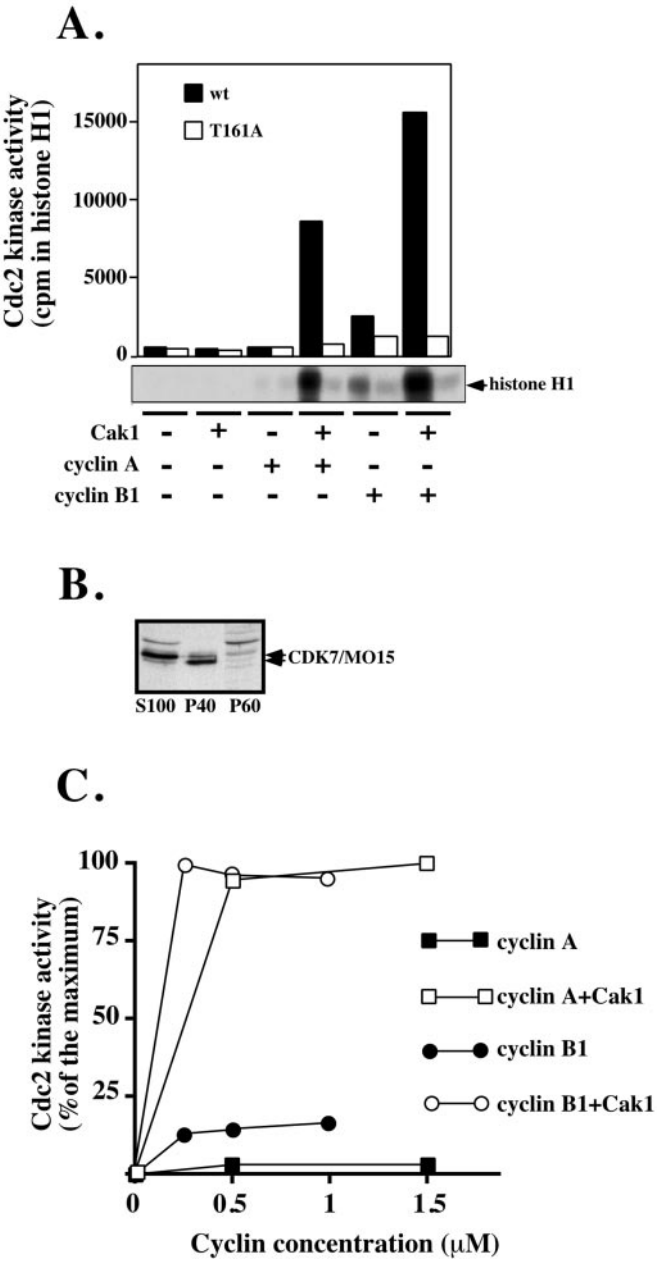

FIG. 2. ***In vitro* activation of Cdc2 by Cak1 and cyclins.** *A*, activation of *Xenopus* recombinant wild type Cdc2 (*wt*) or T161A mutant Cdc2 (*T161A*). The histone H1 activity of recombinant proteins (1.5 μg) was measured *in vitro* after incubation in the presence or not of recombinant Cak1 (0.2 μg) and either His-cyclin B1 or GST-cyclin A (3 μg). *Upper panel*, quantification of Cdc2 kinase activity, expressed in cpm incorporated in histone H1. *Lower panel*, autoradiogram of phosphorylated histone H1. *B*, the 100,000 × *g* (S100) cytosolic extract and the P40 and the P60 ammonium precipitates were analyzed by Western blotting with the anti-MO15/CDK7 antibody. *C*, activation of monomeric Cdc2 in P60. The histone H1 kinase activity of endogenous Cdc2 in P60 (120 μg of protein/assay, equivalent to 10 oocytes/assay) was measured *in vitro* after incubation in the absence or in the presence of Cak1 (0.2 μg) and increasing concentrations of GST-cyclin A or His-cyclin B1. Cdc2 activity is expressed as a percentage of its maximum activity.

F9, which would be active toward the Thr-161 residue of Cdc2 and would counteract Cak1 activity.

To ascertain this hypothesis, we investigated the presence of a phosphatase active toward the activating Thr residue in the T-loop of Cdc2 and Cdk2 (Thr-161 for Cdc2 and Thr-160 for Cdk2). We used soluble bacterial recombinant *Xenopus* GST-Cdk2 that is a better *in vitro* substrate of Cak1 than recombi-

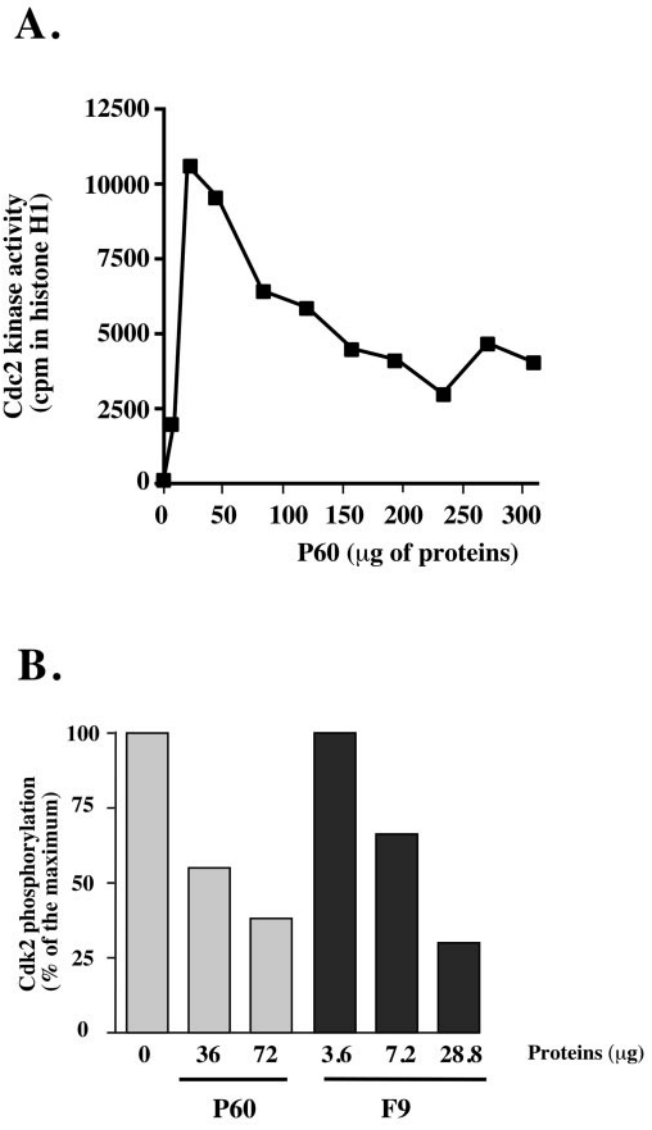

FIG. 3. **A phosphatase present in P60 and F9 counteracts Cdc2 and Cdk2 activation by Cak1.** *A*, increasing amounts of P60 were incubated with Cak1 (0.2 μg) and GST-cyclin A (3 μg or 1.5 μM). The Cdc2·cyclin A complexes were recovered on GSH beads, and the histone H1 kinase activity of Cdc2 was then measured. *B*, recombinant Cdk2 was first phosphorylated by Cak1 in the presence of [γ-^{32}P]ATP. The phosphorylated protein was then incubated for 30 min with increasing amounts of P60 or F9. Phosphorylation of Cdk2 was detected by electrophoresis and autoradiography. The radioactivity incorporated in Cdk2 band was quantified and expressed as a percentage of the maximum.

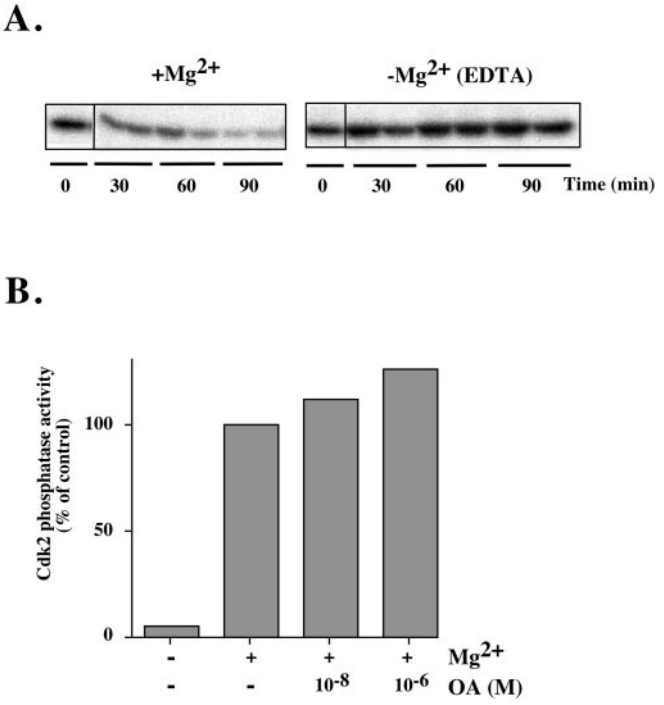

FIG. 4. **Cdk2 phosphorylated by Cak1 is a substrate of a Mg^{2+}-dependent and okadaic acid-insensitive phosphatase present in P60 and in F9.** *A*, recombinant Cdk2 was first phosphorylated by Cak1 in the presence of [γ-^{32}P]ATP. Phosphorylated Cdk2 was then incubated for different times in the presence of P60 prepared in EB, supplemented or not by 30 mM EDTA. The level of Cdk2 phosphorylation was followed by autoradiography. At 30, 60, and 90 min, duplicates are illustrated. *B*, recombinant Cdk2 was first phosphorylated by Cak1 in the presence of [γ-^{32}P]ATP. Phosphorylated Cdk2 was incubated for 20 min with F9, in the absence or the presence of 20 mM Mg^{2+} and two concentrations of okadaic acid (*OA*). The release of ^{32}P from Cdk2 was expressed as a percentage of control in the absence of OA.

nant Cdc2 (10) and can be produced in large amount under a soluble form. Therefore, in a first attempt, Cdk2 was more appropriate than Cdc2 as an *in vitro* substrate to biochemically characterize the phosphatase. GST-Cdk2 was phosphorylated *in vitro* in the presence of Cak1 and [γ-^{32}P]ATP. The ^{32}P-phosphorylated Cdk2 protein was then incubated in the presence of increasing amounts of either P60 or F9 and recovered on GSH beads (Fig. 3*B*). The level of GST-Cdk2 phosphorylation was reduced upon P60 or F9 addition in a dose-dependent manner. This clearly indicates that a phosphatase active toward the Thr-160 residue of *Xenopus* Cdk2 co-purifies with the endogenous monomeric *Xenopus* Cdc2.

Characterization of a Mg^{2+}-dependent Cdk2 Phosphatase Activity in P60 and F9—A preliminary characterization of the Thr-160-Cdk2-specific phosphatase was performed. Because the purification buffer, EB, contains 20 mM EGTA, a Ca^{2+} chelator, the implication of the calmodulin-Ca^{2+}-dependent phosphatase, PP2B, was ruled out. We tested the possibility that the Cdk2 phosphatase belongs to the PP2C family of Mg^{2+}-dependent phosphatases. Cdk2 was *in vitro* phosphorylated by Cak1 and then incubated for different times in P60, in the presence or in the absence of EDTA, a Mg^{2+}/Ca^{2+} chelator. Cdk2 dephosphorylation was analyzed by autoradiography (Fig. 4*A*). In the presence of Mg^{2+} (no EDTA), Cdk2 was efficiently dephosphorylated within 90 min by the phosphatase present in P60 (Fig. 4*A*). In contrast, the absence of Mg^{2+} (addition of EDTA) totally inhibited Cdk2 dephosphorylation (Fig. 4*A*), arguing that the Cdk2 phosphatase activity depends on Mg^{2+}. We further analyzed the Mg^{2+} dependence of the Cdk2 phosphatase in F9. As shown in Fig. 4*B*, F9 contains a Mg^{2+}-dependent phosphatase active toward Thr-160-phosphorylated Cdk2. Okadaic acid, a well-known inhibitor of PP2A and PP1 (28), did not affect the dephosphorylation of Thr-160-phosphorylated Cdk2 by the phosphatase present in F9 (Fig. 4*B*). Altogether, these results show that P60 and F9 contain a Mg^{2+}-dependent phosphatase, which could antagonize Cak1 activity on Thr-161 residue of Cdc2.

Isolation and Cloning of a Xenopus Phosphatase 2C That Dephosphorylates Cdk2 on Thr-160—The Mg^{2+} dependence of the Cdk2 phosphatase present in P60 and F9 strongly suggests that it could belong to the PP2C family. To go further into the molecular characterization of this phosphatase, a purification procedure was undertaken. Two substrates were used to follow the activity: GST-Cdk2 phosphorylated on Thr-160 by Cak1 and α/β-casein phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase, PKA. At each step of the

TABLE 1
Isolation of Cdk2 phosphatase from Xenopus ovaries

^{32}P-Thr-160-phosphorylated Cdk2 protein was used as substrate, and the assays were performed in the presence of 20 mM Mg^{2-}. One unit of Cdk2 phosphatase corresponds to 1 pm of orthophosphate released per minute at 30 °C under standard conditions.

Fraction	Total proteins	Total activity	Specific activity	Recovery	Purification
	mg	*milliunits*	*milliunits/mg*	*%*	*fold*
Ovary extract	6000	NM[a,b]	ND	ND	
P60	1500	30,000[b]	20	100	1
DEAE-Scpharosc	660	45,000	68	ND	3.37
S200	100	10,200	100	35	5
UnoQ	22	7,815	365	26	17.5
Phenyl-Superose	0.5	900	1800	2.5	90
Hi-Trap Blue	<0.1	542	5425	1.8	262.5

[a] ND, not determined; NM, nonmeasurable.
[b] In the ovary extract as well as in P60, the Cdk2 phosphatase activity is not linearly proportional with the amount of total proteins. This observation suggests that the phosphatase could be in a partially inhibited state in these fractions.

purification procedure, fractions were assayed using both substrates in the presence or in the absence of 10^{-6} M okadaic acid plus or minus Mg^{2+}. A P60 ammonium precipitate was prepared from *Xenopus* ovaries as starting material. The Mg^{2+}-dependent Cdk2 phosphatase activity was further purified by DEAE-Sepharose anion exchange, gel filtration (Sephacryl S200), anion exchange (UnoQ), hydrophobic interaction (Phenyl-Superose), and affinity chromatography (Hi-Trap Blue) (Table I). PP2C was detected by Western blot using an anti-human PP2Cα antibody in all the active fractions recovered after each step of the purification procedure (Fig. 5*A*). The last step gave raise to a fraction containing a Cdk2 and casein phosphatase activity dependent on Mg^{2+} and insensitive to okadaic acid (Table I, Fig. 5*B*, and data not shown). After electrophoretic separation, a faint 45-kDa band was detected by Amido Black staining (Fig. 5*A*). Western blotting confirmed the presence of a 45-kDa PP2C protein in this fraction (Fig. 5*A*) whereas PP2A subunits and PP1 catalytic subunit were undetectable (data not shown).

Xenopus PP2Cα cDNA was then cloned from a *Xenopus* oocyte cDNA library and sequenced (EMBL data base accession number AJ438209). The deduced amino acid sequence of the protein exhibits about 89% identity with its mammalian counterparts, indicating that the protein is highly conserved among species. *Xenopus* PP2Cα was then subcloned in the pThioHisB bacterial expression vector and produced in *E. coli*. After purification, the activity of the recombinant protein was assayed using Cdk2 phosphorylated by Cak1 and casein phosphorylated by PKA as substrates. The recombinant protein exhibits a casein phosphatase activity highly dependent on Mg^{2+} and insensitive to okadaic acid (Fig. 6*A*). Dephosphorylation of Cdk2 was analyzed by two methods: first in a standard phosphatase assay, monitoring ^{32}P release from phosphorylated Cdk2 (Fig. 6*A*); second, by following the Thr-160 phosphorylation level of Cdk2 by using on Western blot an antibody recognizing specifically the activating phospho-Thr residue in the CDK T-loop (Fig. 6*B*). Both assays showed that recombinant PP2C is able to *in vitro* dephosphorylate Cdk2 on Thr-160 in a Mg^{2+}-dependent manner (Fig. 6, *A* and *B*). Addition of cyclin A to phosphorylated Cdk2 abolished the phosphatase activity of recombinant PP2C (Fig. 6*C*), whereas the presence of cyclin A did not affect PP2C activity toward casein (data not shown). This indicates that the cyclin-bound form of Cdk2 is not a substrate of PP2C. Altogether, our results show that the *Xenopus* phosphatase able to dephosphorylate the activating Thr-160 residue of the T-loop of monomeric Cdk2 is PP2C.

A Subpopulation of Endogenous Monomeric Cdc2 Is Phosphorylated on Thr-161—PP2C copurifies with monomeric Cdc2. Therefore, if monomeric Thr-161-phosphorylated Cdc2 is present in the oocyte, it would probably be dephosphorylated by

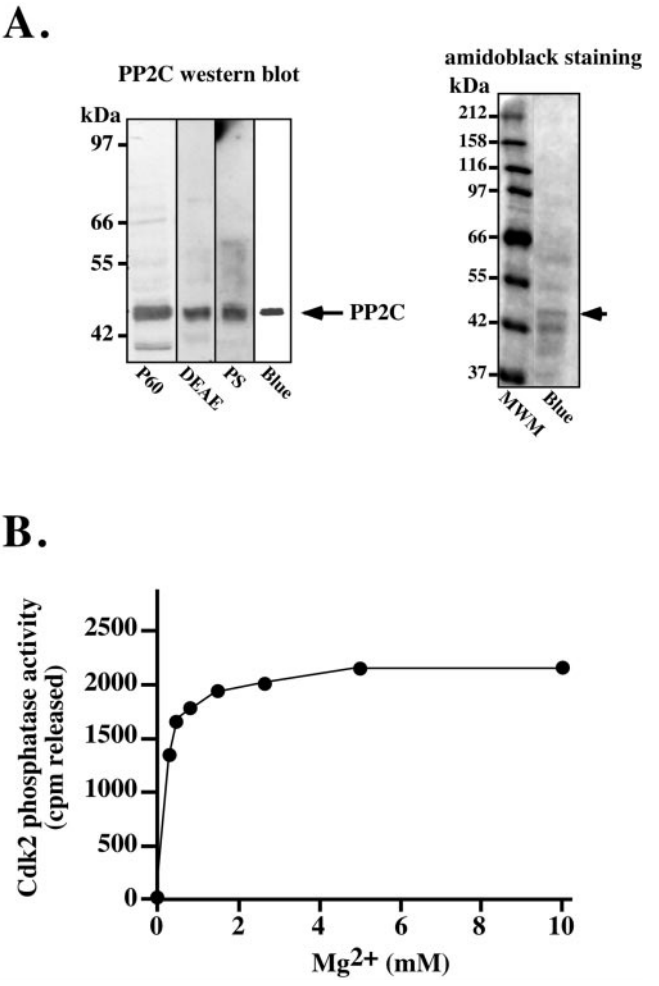

FIG. 5. **Purified *Xenopus* PP2C dephosphorylates the Thr-160 residue of Cdk2.** *A*, *Xenopus* PP2C was purified according the procedure described in Table I. The active fractions of various purification steps (P60, DEAE-Sepharose, Phenyl-Superose: *PS*; Hi-Trap Blue: *Blue*) were pooled, electrophoresed, and Western-blotted with the anti-PP2C antibody (*left panel*). Proteins from the active fraction of the last purification step (Hi-Trap Blue column) were visualized by Amido Black staining (*right panel*). *MWM*, molecular weight markers. *B*, recombinant Cdk2 was first phosphorylated by Cak1 in the presence of [γ-^{32}P]ATP, then incubated for 20 min in the presence of 0.1 μg of the active fraction of the last purification step (Hi-Trap Blue column) and increasing concentrations of Mg^{2+}, and the release of ^{32}P from Cdk2 was measured.

this phosphatase during the homogenization step and the purification procedure. Indeed, addition of GST-cyclin A to P60 or F9 prepared in the presence of Mg^{2+} (EB) was not sufficient to

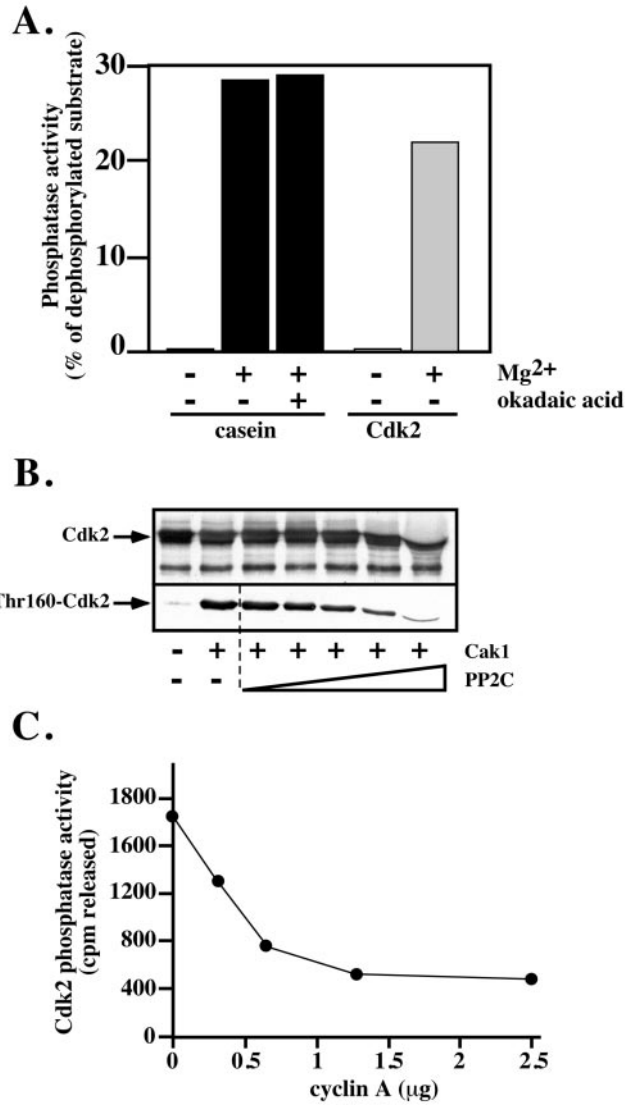

FIG. 6. ***Xenopus* recombinant PP2C dephosphorylates the Thr-160 residue of Cdk2.** *A*, recombinant Cdk2 and α/β casein were first phosphorylated in the presence of [γ-^{32}P]ATP by Cak1 and PKA, respectively. Phosphorylated Cdk2 or casein were then incubated for 20 min in the presence of 2 μg *Xenopus* recombinant PP2C protein, in the presence or in the absence of Mg^{2+} and 10^{-6} M okadaic acid, and the percentage of dephosphorylated substrate was measured. *B*, recombinant Cdk2 (5 μM) was first phosphorylated by Cak1 in the presence of cold ATP and then incubated for 30 min at 30 °C with increasing concentrations (1.2, 0.24, 0.12, 0.024, and 0.012 mg/ml) of *Xenopus* recombinant PP2C. Proteins were Western-blotted with an antibody recognizing specifically the phospho-Thr-160 residue of Cdk2 (*lower panel*) and an anti-PSTAIR antibody recognizing all forms of Cdk2 (*upper panel*). *C*, recombinant Cdk2 was first phosphorylated by Cak1 in the presence of [γ-^{32}P]ATP. Phosphorylated Cdk2 was incubated for 30 min in the presence of increasing concentrations of recombinant cyclin A. *Xenopus* recombinant PP2C (2 μg) was then added in the presence of Mg^{2+}, and the release of ^{32}P from Cdk2 was measured.

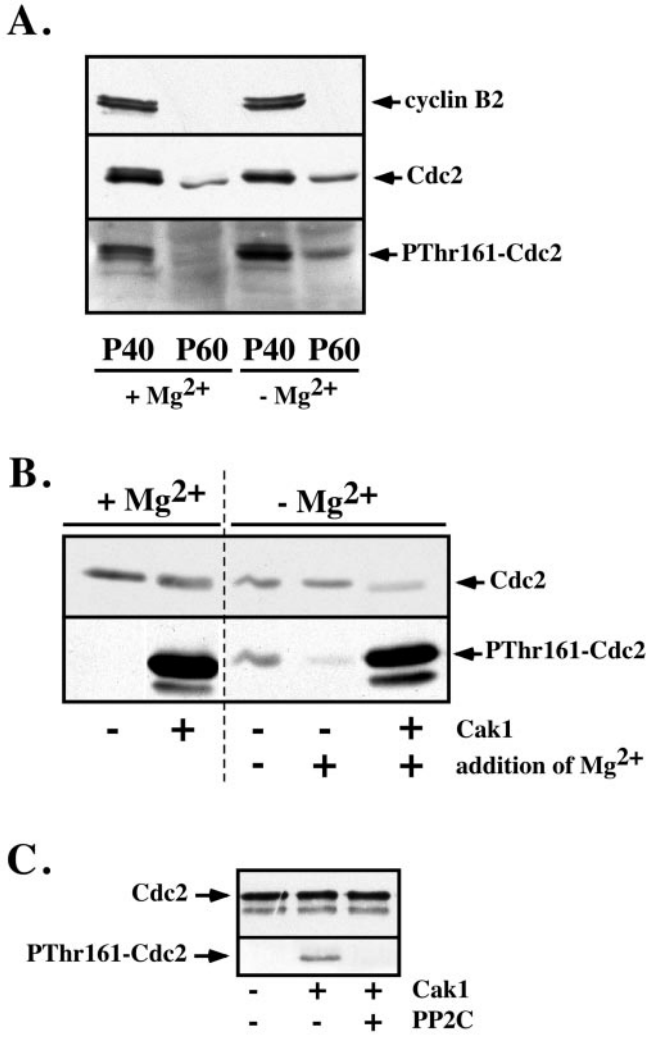

FIG. 7. **Monomeric Cdc2 in P60 and F9 is phosphorylated on Thr-161.** *A*, P40 and P60 were prepared in the absence ($-Mg^{2+}$) or in the presence of Mg^{2+} ($+Mg^{2+}$). They were analyzed by Western blotting with the anti-cyclin B2 antibody (*upper panel*), the anti-Cdc2 antibody (*middle panel*), and the antibody recognizing specifically the phospho-Thr-161 residue of Cdc2 (*lower panel*). *B*, F9 was prepared in the absence ($-Mg^{2+}$) or in the presence of Mg^{2+} ($+Mg^{2+}$). As indicated, 25 mM Mg^{2+} with or without Cak1 was added back or not in F9 initially prepared in the absence of Mg^{2+}, and the fraction was further incubated for 30 min at 30 °C before Western blotting with the anti-Cdc2 antibody (*upper panel*) and the antibody recognizing specifically the phospho-Thr-161 residue of Cdc2 (*lower panel*). *C*, F9 was prepared in the presence of Mg^{2+} and then incubated in the presence or in the absence of Cak1 for 30 min at 30 °C. PP2C (0.9 mg/ml in the final assay) was added or not, and then incubation was extended for 30 min. Samples were analyzed by Western blotting with the anti-Cdc2 antibody (*upper panel*) and the antibody recognizing specifically the phospho-Thr-161 residue of Cdc2 (*lower panel*).

activate histone H1 kinase in the absence of Cak1 (Fig. 2*B*). To determine whether some monomeric subpopulation of Cdc2 was phosphorylated on Thr-161, oocyte fractions (P40, P60, and F9) were prepared in the presence or in the absence of Mg^{2+}. For this purpose, two buffers were used: either EB (20 mM EGTA and 15 mM $MgCl_2$) or modified EB (no EGTA, no $MgCl_2$, and 10 mM EDTA). The Thr-161-phosphorylated form of Cdc2 was analyzed by Western blot, using the specific antibody recognizing the phospho-Thr-161 residue of Cdc2. This antibody detected a strong band in P40 (Fig. 7*A*), and this band included most probably cyclin B-associated Cdc2 (pre-MPF). Interestingly, Thr-161-phosphorylated Cdc2 was detected in P60 and F9, both enriched in monomeric Cdc2, when prepared in the absence of Mg^{2+} (Fig. 7, *A* and *B*). In the presence of Mg^{2+} during the fraction preparation, Thr-161 phosphorylation of monomeric Cdc2 was undetectable (Fig. 7, *A* and *B*).

F9 prepared in the absence of Mg^{2+} to preserve some Thr-161 phosphorylation level of Cdc2 was then supplemented with Mg^{2+}. After a 30-min incubation at 30 °C, the content of Thr-161-phosphorylated Cdc2 was analyzed. Addition of Mg^{2+} in F9 strongly diminished Thr-161 phosphorylation of Cdc2 (Fig. 7*B*). In a reciprocal experiment, addition of Cak1 in F9 led to a strong increase in the level of Thr-161-phosphorylated Cdc2 (Fig. 7*B*). Altogether, these results show that monomeric Cdc2

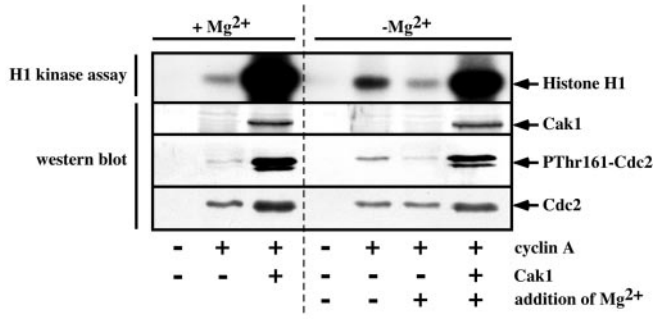

FIG. 8. **The subpopulation of monomeric Thr-161-phosphorylated Cdc2 is activable by cyclin.** P60 was prepared in the absence ($-Mg^{2+}$) or in the presence of Mg^{2+} ($+Mg^{2+}$) and incubated in the presence or in the absence of GST-cyclin A (3 μg) and Cak1 (0.2 μg) in modified EB buffer (EDTA). As indicated, Mg^{2+} was added back or not in P60 initially prepared in the absence of Mg^{2+}. Cdc2·cyclin A complexes were recovered by GST pull-down. The pull-down samples were either assayed for histone H1 kinase activity (*top panel*) or Western-blotted with the following antibodies: anti-Cak1, anti-phospho-Thr-161 Cdc2, and anti-Cdc2 antibodies.

is partially phosphorylated on Thr-161 and that an endogenous Mg^{2+}-dependent phosphatase 2C actively dephosphorylates this residue in P60 and F9.

We then confirmed that monomeric Thr-161-phosphorylated Cdc2 is a substrate of *Xenopus* PP2C. F9 was first incubated in the presence of purified Cak1, leading to the phosphorylation of endogenous monomeric Cdc2 (Fig. 7*C*). Then, recombinant PP2C was added, inducing a total dephosphorylation of Cdc2 (Fig. 7*C*) and demonstrating that Thr-161 residue of *Xenopus* Cdc2 is dephosphorylated by PP2C.

Monomeric Cdc2 Phosphorylated on Thr-161 Is Directly Activable by Cyclin Binding—Because monomeric Cdc2 is phosphorylated to some extent on Thr-161, it should be directly activated by cyclin binding. To ascertain this hypothesis, GST-cyclin A was added to P60, prepared with and without Mg^{2+}, in the absence of Cak1. The GST·cyclin A·Cdc2 complexes were then recovered on GSH beads and Cdc2 kinase activity was assayed. Fig. 8 represents a typical experiment. As expected, in the presence of Mg^{2+}, Cdc2 phosphorylation on Thr-161 was nearly undetectable, and cyclin A addition did not stimulate any Cdc2 kinase activity (Fig. 8). In contrast, in the absence of Mg^{2+}, phospho-Thr-161 was clearly detected and correlated with a reproducible activation of Cdc2 kinase after cyclin addition (Fig. 8). Interestingly, addition of Mg^{2+} to P60 initially prepared in the absence of Mg^{2+} to preserve Thr-161 phosphorylation of Cdc2 led to Thr-161 dephosphorylation and to a parallel decrease of its activation by cyclin (Fig. 8). The activation of monomeric Cdc2 by cyclin binding therefore directly reflects its phosphorylation level on Thr-161, which is under the control of PP2C activity.

To roughly estimate the proportions of Thr-161-phosphorylated monomeric Cdc2 *versus* unphosphorylated monomeric Cdc2, the following experiment was performed. P60 was prepared in the absence of Mg^{2+}, to preserve the Thr-161-phosphorylation of Cdc2. GST-cyclin A was then added in the presence or in the absence of Cak1. Cdc2·cyclin A complexes were recovered by cyclin binding on GSH beads, and their kinase activity was measured. Under these conditions, Cak1 was also recovered on GSH beads (Fig. 8), indicating that it has a strong affinity for Cdc2 complexes, in agreement with the previous observations of Thuret and colleagues (10). Interestingly, the amounts of cyclin A·Cdc2 recovered by GSH beads were higher in the presence of Cak1 (Fig. 8), suggesting that the phosphorylation of Cdc2 on Thr-161 stabilizes its association with cyclin, as previously suggested (29). Cdc2 activation generated by addition of cyclin alone allowed us to measure the level of Cdc2 already phosphorylated on Thr-161, whereas the addition of cyclin A together with Cak1 allowed the activation of all Cdc2 molecules of the fraction. In this typical experiment, Cdc2 activation by cyclin was about 10-fold higher in the presence of Cak1 than without Cak1 (Fig. 8). We estimated that Cdc2 activation by cyclin without Cak1 represents 7.2 ± 1.1% ($n = 3$) of the activity generated in the presence of Cak1, indicating that Thr-161-phosphorylated Cdc2 represents about 7% of the monomeric Cdc2 molecules present in P60 under our experimental conditions. This amount of Thr-161-phosphorylated monomeric Cdc2 would be of the same order of magnitude than the amount of inactive cyclin B-bound Cdc2 in the oocyte, *i.e.* pre-MPF, as estimated by Kobayashi and colleagues (1).

DISCUSSION

MPF or Cdc2 protein kinase drives *Xenopus* oocyte meiotic maturation. It is regulated by the availability of cyclin subunits and phosphorylation/dephosphorylation reactions. The activating phosphorylation on Thr-161 within the T-loop is required for kinase activity (5) and should be regulated at different critical phases of oogenesis: first, during pre-MPF accumulation occurring during the last period of oocyte growth; second at the entry into metaphase I (or GVBD) when MPF is first activated; and third, during the metaphase I-metaphase II transition when cyclin turnover occurs. Does the addition of a phosphate to Thr-161 of Cdc2 precede or follow cyclin binding at each of these steps? Our results show that a significant fraction of monomeric Cdc2 partially purified in a Mg^{2+}-free buffer from prophase resting oocytes is directly activable *in vitro* by cyclin addition. This result indirectly suggests that Thr-161-phosphorylated free Cdc2 is present in the oocyte. We directly evidenced the presence of this phosphorylated form of Cdc2 by using an antibody specifically directed against Thr-161-phosphorylated Cdc2. This antibody is able to recognize monomeric Cdc2, only when prepared in the absence of Mg^{2+}. To ascertain the specificity of the antibody, recombinant *Xenopus* Cdk2 or partially purified *Xenopus* monomeric Cdc2 (F9) were phosphorylated *in vitro* on Thr-160 or Thr-161, respectively, by recombinant Cak1. Western blots illustrated in Figs. 6 and 7 clearly demonstrate the high detection specificity of the phosphorylated activating Thr of the T-loop of Cdk2 and Cdc2 by this antibody. Therefore, through two distinct experimental approaches, it is possible to conclude that monomeric Thr-161-phosphorylated Cdc2 is present in the oocyte where it represents a latent form of Cdc2 directly activable by cyclin binding. Consequently, one might postulate that *Xenopus* oocyte contains the enzymes that control the addition and removal of phosphate on residue Thr-161 of monomeric Cdc2. Mg^{2+} chelation in purification buffers is required for the immunodetection of phosphorylated monomeric Cdc2, which appears to be directly activable by cyclin binding. This prompted us to search for the presence of a Mg^{2+}-dependent protein phosphatase specific of Thr-161-phosphorylated Cdc2 in the *Xenopus* oocyte. A partial purification led to the isolation of a 45-kDa Mg^{2+}-dependent phosphatase, which is recognized by an antibody raised against human PP2Cα and exhibits the enzymatic properties of PP2C. This purified enzyme was able to dephosphorylate recombinant Thr-160-phosphorylated Cdk2 as well as Thr-161-phosphorylated *Xenopus* Cdc2. After bacterial expression, the recombinant *Xenopus* PP2Cα similarly possesses a Mg^{2+}-dependent phosphatase activity able to dephosphorylate the Thr-161 residue of monomeric Cdc2. When cyclins are added to prephosphorylated Cdc2, PP2C is not able to dephosphorylate Thr-161 anymore, indicating that cyclin·Cdc2 complex is not a PP2C substrate.

In a previous study, Poon and Hunter (30) reported that an EDTA-treated extract prepared from *Xenopus* eggs contains a "KAP"-like activity that dephosphorylates monomeric Thr-160-phosphorylated recombinant Cdk2. This phosphatase activity present in egg extracts has not been further characterized. Under our experimental conditions, no KAP-like phosphatase could be found in extracts from prophase oocytes prepared in the presence of EDTA. An intriguing possibility, which remains to be experimentally explored, could be that a KAP-like phosphatase activity, absent or inactive in prophase oocyte, is neosynthetized or unmasked during meiotic maturation.

The copurification of a PP2C-like phosphatase with monomeric Cdc2 explains why Thr-161-phosphorylated Cdc2 had not been previously identified in *Xenopus* oocytes. Indeed, the standard EB buffer used to isolate pre-MPF or MPF contains a high Mg^{2+} concentration (15 mM) (24), allowing full activity of Mg^{2+}-dependent phosphatases and leading consequently to dephosphorylation of Cdc2.

Solomon and co-workers (31) identified genetically and biochemically Ptc2p and Ptc3p in *S. cerevisiae* as the two major type 2C phosphatases that dephosphorylate monomeric CDC28. Therefore, PP2C physically opposes the biological functions of monomeric Cak1 in budding yeast. Human HeLa cells also contain two PP2C isoforms, PP2Cα and β2, that dephosphorylate monomeric human Cdk2/Cdk6 *in vitro* (32). These new observations raise, by analogy, the possibility that phosphorylated monomeric Cdc2 isolated from *Xenopus* oocyte could also be regulated by a monomeric CAK and PP2C. Whereas our results establish that a phosphatase 2C catalyzes the removal of phosphate on Thr-161/Thr-160 of Cdc2/Cdk2, it is at present uncertain whether the *Xenopus* oocyte contains an enzyme that catalyzes the phosphorylation of Thr-161 in monomeric Cdc2. Identification of monomeric Cdc2 phosphorylated on Thr-161 together with the low affinity of CDK7·cyclin H enzyme for monomeric CDKs (9) favors the view that such an enzyme would be present and functional in the oocyte. A difficulty encountered for the purification of this putative kinase is to inhibit or to remove the Mg^{2+}-dependent phosphatase activity that opposes to this kinase activity.

Our results show for the first time that monomeric Thr-161-phosphorylated Cdc2 can be isolated from *Xenopus* extracts and that it is a substrate of an endogenous PP2C. A specific regulation, implying the Thr-161 kinase and/or PP2C, allows the presence of two monomeric Cdc2 subpopulations in the oocyte, one being phosphorylated on Thr-161 and directly activable by cyclin binding while the other one is not. These results have important physiological implications. Of particular interest is the possible role of phosphorylated monomeric Cdc2 in the initiation of the MPF autoamplification loop. A small increase in cyclin B availability might be sufficient to bind with and to activate Cdc2 already phosphorylated on Thr-161 and then to generate a threshold Cdc2 kinase activity able to trigger MPF autoamplification. A recent study, using an antisense strategy, reported that the synthesis of cyclins B1, B2, B4, and B5 is not required *in ovo* for the initiation of MPF amplification during oocyte maturation (2). It cannot be excluded, however, that beyond cyclins B, another cyclin or an unknown partner of Thr-161-phosphorylated monomeric Cdc2 could be involved in the switching on of its kinase activity. A major objective will be to determine the levels of Thr-161-phosphorylated monomeric Cdc2 and to study how the phosphatase 2C and its opposed kinase are subject to regulation during the whole meiotic maturation process.

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