VERSITA

CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 18 March 2011
Final form accepted: 16 September 2011
Published online: 26 September 2011

Volume 16 (2011) pp 669-688
DOI: 10.2478/s11658-011-0030-z

Research article

INHIBITOR-2 INDUCED M-PHASE ARREST IN *XENOPUS* CYCLING EGG EXTRACTS IS DEPENDENT ON MAPK ACTIVATION

ARIAN KHANDANI[1]*, MAHMOOD MOHTASHAMI[2] and ANNE CAMIRAND[3]

[1]Department of Cell Biology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7, [2]Department of Immunology, University of Toronto and Sunnybrook Research Institute, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5
[3]Lady Davis Institute for Medical Research, McGill University, Montréal, Québec, Canada H3T 1E2

Abstract: The evolutionarily-conserved protein phosphatase 1 (PP1) plays a central role in dephosphorylation of phosphoproteins during the M phase of the cell cycle. We demonstrate here that the PP1 inhibitor inhibitor-2 protein (Inh-2) induces an M-phase arrest in *Xenopus* cycling egg extracts. Interestingly, the characteristics of this M-phase arrest are similar to those of mitogen-activated protein kinase (p42MAPK)-induced M-phase arrest. This prompted us to investigate whether Inh-2-induced M-phase arrest was dependent on activation of the p42MAPK pathway. We demonstrate here that MAPK activity is required for Inh-2-induced M-phase arrest, as inhibition of MAPK by PD98059 allowed cycling extracts to exit M phase, despite the presence of Inh-2. We next investigated whether Inh-2 phosphorylation by the MAPK pathway was required to induce an M-phase arrest. We discovered that while p90Rsk (a MAPK protein required for M-phase arrest) is able to phosphorylate Inh-2, this phosphorylation is not required for Inh-2 function. Overall, our results suggest a novel mechanism linking p42MAPK and PP1 pathways during M phase of the cell cycle.

* Author for correspondence. e-mail:arian.khandani@sickkids.ca

Abbreviations used: CC – chromosome condensation; CSF – cytostatic factor; DIC – differential interference contrast; DMSO – dimethyl sulfoxide; EGS – ethylene glycol-bis(succinic acid *N*-hydrosysuccinimide ester); Inh-2 – inhibitor-2; MAPK – mitogen-activated protein kinase; MEK – MAPK/extracellular signal-regulated kinase kinase; NEBD – nuclear envelope breakdown; PKA – protein kinase A ; p90Rsk-1 – p90 ribosomal S6 kinase-1; SAC – spindle assembly checkpoint

Key words: Inhibitor-2, PP-1 phosphatase, MAPK kinase, p90Rsk, M-phase arrest, *Xenopus* cycling egg extract

INTRODUCTION

The amphibian system, specifically the *Xenopus laevis* system, is a well-established model for studying transitions through the cell cycle and the molecular pathways that regulate such events. The *Xenopus* oocytes are naturally arrested at the G2 phase of the cell cycle and can be released to enter M phase either by natural or artificial means. Progression through the cell cycle in oocytes was first shown to be induced by maturation promoting factor (MPF) [1], whose activity is known to oscillate during the cell cycle, with its highest activity during M phase. The two components of MPF, identified as Cdc2 and Cyclin B, belong respectively to the cyclin-dependent kinase cell division cycle (Cdc), and to the cyclin families of proteins, which are collectively the major regulators of cell cycle progression.

Another level of regulation imposed during cell cycle progression is the activity of the p42/44 mitogen-activated protein kinase (MAPK) pathway. The p42 isoform (ERK2) has been extensively characterized for its role in many aspects of *Xenopus* cell cycle regulation [2], including maturation, M-phase arrest of the unfertilized eggs (referred to as cytostatic factor or CSF arrest), and spindle assembly checkpoint (SAC). Activation of the MAPK pathway can induce an M-phase arrest in *Xenopus* cycling egg extracts [3-8]. Within the MAPK pathway, p90 ribosomal S6 kinase-1 (p90Rsk-1) is the downstream kinase required for CSF and the induction of M-phase arrests in *Xenopus* cycling egg extracts [8, 9]. While direct targets of p90Rsk-1 during the CSF arrest have been identified, the candidate/s for its downstream effects during mitosis (induced M-phase arrests in *Xenopus* cycling egg extracts) remain unknown.

PP1 is a member of a larger family of ser/thr protein phosphatases that are highly conserved among eukaryotes [10, 11]. A number of observations have suggested that PP1 is involved during exit from M phase. For instance, temperature-sensitive mutations in PP1 have resulted in M-phase defects such as aberrations in chromosomal separation and undivided nuclei in both fission and budding yeasts [12-16]. In addition, recent evidence showed that PP1 is also required for spindle assembly checkpoint during M phase in both fission and budding yeasts [17, 18]. In higher eukaryotes, microinjection of PP1 antibodies either during entry or in mid-M phase resulted in M-phase arrest in rat embryo fibroblasts [19]. Inhibition by okadaic acid after inactivation of Cdc2 kinase induced an M-phase arrest in *Xenopus* mitotic extracts [20]. The assortment of defects caused by PP1 inhibition can be explained by its interaction with several different regulatory subunits. PP1 catalytic subunits (α, β, and γ isoforms) are rarely found isolated from their various regulatory subunits [10, 21], which include inhibitor-2 (Inh-2).

Inh-2 is a heat-stable 23-kDa protein that inhibits PP1 activity in its unphosphorylated state [10, 21, 22]. Many kinases have been shown to regulate PP1 activity through phosphorylation of Inh-2 *in vitro*. For instance, glycogen synthase kinase-3 (GSK3), MAPK, and Cdc2 kinases all phosphorylate Inh-2 on Thr 72 residue and activate the Inh-2/PP1 complex [23-28]. However, it has been shown that Cdc2 kinase is mainly responsible for Inh-2 phosphorylation of Thr 72 residue during M phase in HeLa cells [29, 30]. In addition, p90Rsk phosphorylates Inh-2 protein *in vitro*, but this phosphorylation does not appear to affect the phosphatase activity of PP1[26]. Expression of Inh-2 protein oscillates in rat fibroblasts, with high levels during M and S phases, similar to that of PP1 [31]. In *Xenopus* cycling egg extracts, Inh-2 addition at concentrations that inhibited PP1 activity induced early entry into M phase [32], whereas its immunodepletion delayed entry into M phase [33]. In addition, knockdown of Inh-2 by siRNA induced mitotic defects in human adult retinal epithelial cells [34].

Addition of either Inh-2 or Inh-1 (which is another inhibitory subunit of PP1) to CSF arrested extracts was recently reported to sustain the M-phase arrest after Cdc2 inactivation by roscovitine [20]. It was also shown that protein kinase A (PKA) was responsible for the phosphorylation of Inh-1 that is partially required for the induced M-phase arrest. In this paper, we investigate the role of Inh-2 protein and show that it is able to induce a similar M-phase arrest in *Xenopus* cycling egg extracts. Since Inh-2 protein does not contain the PKA phosphorylation consensus site, we wanted to further investigate if other pathways regulate the Inh-2-induced M-phase arrest. We show in this report that the Inh-2-induced M-phase arrest is dependent on p42MAPK pathway activation in *Xenopus* cycling egg extract.

MATERIALS AND METHODS

All reagents were purchased from Sigma or VWR unless otherwise indicated.

Preparation of recombinant histidine-tagged Inh-2 protein

The rabbit Inh-2 cDNA was subcloned into the pET28a plasmid and expressed in *Escherichia coli* (*E. coli*). The bacterial culture was grown using standard procedures, then induced with 1 mM isopropylthio-β-D-galactoside (IPTG) at log phase growth and incubated under the same conditions for 4 hrs. The cells were pelleted and resuspended in lysis buffer (25 mM Na_2HPO_4-NaH_2PO_4, pH 7.5, 125 mM NaCl, 1.0% Tween-20, 10 μg/ml each of leupeptin, chymostatin, and pepstatin, 2.0 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF]), frozen using liquid nitrogen and thawed (twice), followed by sonication on ice (3x 20 pulse sets at 6 output, 30% duty, separated by 1 min using the broad probe). After centrifugation, the supernatant (pre-binding sample) was removed and added to Ni^{2+}-charged nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen Inc) for 1 hr and mixed end-over-end at 4°C. After binding, the beads underwent numerous washes in lysis buffer (without protease inhibitors) containing 1.0%

Tween-20 at 4°C, followed by once with lysis buffer with 1.0% Tween-20 and 0.5 M NaCl, and twice with lysis buffer with 1.0% Tween-20. The next series of washes were done at room temperature: 3 times with lysis buffer and twice with final wash buffer (25 mM Na_2HPO_4-NaH_2PO_4, pH 7.5, 125 mM NaCl, 20 mM imidazole). The His-tagged Inh-2 protein was eluted with increasing concentrations of imidazole (50 to 250 mM) in dilution buffer (25 mM Na_2HPO_4-NaH_2PO_4, pH 7.5, 125 mM NaCl). The eluted fractions that contained His-tagged Inh-2 protein were pooled and dialyzed against dialysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [HEPES]-KOH, pH 7.0, 5 mM $MgCl_2$, 20% glycerol) at 4°C, using 3,500 molecular weight cut-off dialysis tubing (Fisher Scientific). The protein was concentrated against the same buffer containing 10% polyethylene glycol (M.W. 20,000) at 4°C. The concentration was then estimated by separating samples on SDS-PAGE followed by staining with Coomassie Blue and comparing it to BSA standard.

Preparation of *Xenopus* egg extracts

Induction of ovulation and dejellying eggs

Female *Xenopus* frogs were injected in the dorsal lymph sac with 100 I.U. of pregnant mare's serum (PMS) (follicle-stimulating hormone activity). After 48 hrs, 1000 I.U. of human chorionic gonadotropin (HCG) (luteinizing hormone) were injected, and frogs were transferred to vessels containing 0.1 M NaCl solution. Within 12-14 hrs after HCG injection, spawned eggs and eggs obtained by gently squeezing the female frog were collected. Eggs were dejellied by placing them in 0.02 g/ml of L-cysteine (hydrochloride) with 0.16 M NaOH, pH 8.1-8.3, and then washed at least 10 times with egg washing solution (0.01 M HEPES-NaOH, pH 7.0, 0.1 M NaCl), followed by examination under the dissection microscope to remove any activated or degenerated eggs.

Cycling egg extract

For cycling egg extracts, dejellied eggs were electrically activated (2 one-second shocks of 15 volts AC, separated by 5 seconds) in 20% Steinberg's solution [11.6 mM NaCl, 0.134 mM KCl, 0.08 mM $MgSO_4.7H_2O$, 0.68 mM $Ca(NO_3)_2.4H_2O$, 0.92 mM Tris (hydroxyamino) methane, 0.8 mM HCl, 0.03 mg/ml penicillin G, 0.05 mg/ml streptomycin sulfate, 0.1 mg/ml sulfathiazole, pH 7.4]. The activation of eggs was monitored by contraction of the animal hemisphere (brown pigment) of eggs, occurring within 3-10 min of the pulse. Just prior to making the extract, activated eggs were washed 3 times in extraction buffer (10 mM HEPES-KOH, pH 7.7, 50 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$) followed by washing twice with the extraction buffer plus protease inhibitors (10 μg/ml of pepstatin, leupeptin, and chymostatin). Activated eggs were transferred to a 3-ml Ultraclear centrifuge tube (Beckman Coulter, Inc.) containing 0.6 ml of silicon oil (Versalube F-50) and overlaid with 0.6 ml of extraction buffer with protease inhibitors and 100 μg/ml of cytochalasin B. The eggs were packed by centrifugation at 1,060xg for 1 min

at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge and excess oil and buffer were aspirated. The packed eggs were crushed by centrifugation (25-35 min after activation) at 10,200xg for 15 min at 4°C using the same rotor, and the cytoplasmic layer was transferred to an ice-chilled 1.6-ml microfuge tube for another round of centrifugation at 10,200xg for 15 min at 4°C. The cleared cytoplasm was removed and 1x volume of an ATP-regenerating system (40 mM adenosine triphosphate [ATP]/Mg^{2+} [Roche], 0.4 M creatine phosphate [Roche], 2 mg/ml creatine kinase [Roche]) was mixed with 37x volumes of the extract. Demembranated sperm nuclei were added to a final concentration of 75-100 nuclei/µl to the extract.

Interphase-arrested egg extracts

Dejellied eggs were incubated in 20% Steinberg's solution with 100 µg/ml of cycloheximide for 7-10 min and then electrically activated and washed 3 times in extraction buffer with 100 µg/ml of cycloheximide, and twice with extraction buffer containing protease inhibitors. The activated eggs were crushed 32-37 min after activation. The extract was obtained following centrifugation steps, and addition of the ATP-regenerating system mentioned above. The extract was then used immediately or frozen in liquid nitrogen in aliquots of 50 µl and stored at -80°C.

M-phase-arrested egg extracts

To obtain M-phase-arrested unfertilized egg extracts, dejellied eggs were washed 3 times in cytostatic factor (CSF) extraction buffer [10 mM HEPES-KOH, pH 7.7, 50 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*,*N*'-tetraacetic acid (EGTA), 20 mM NaF] and twice with the CSF extraction buffer containing protease inhibitors. The egg extract was made in the same way as described above with the exception that cytochalasin B was added after the first centrifugation to a final concentration of 50 µg/ml.

Cycling egg extract assays

Cycling *Xenopus* egg extracts were aliquoted [5], and either rabbit Inh-2 protein (Calbiochem) (or His-tagged Inh-2), human Inh-2, or buffer was added at 5%-14% (v/v) to the reaction (actual additions are specified in the legends). The Inh-2 protein was added to a final concentration of approximately 2.6-2.1 µM for each reaction. The inhibitor PD98059 (Calbiochem) was added to reactions to a final concentration of 300 µM (1: 167 dilution). The final volume of each reaction was 70 µl. The reactions were then incubated in a water bath at 21-23°C and samples for immunoblot, histone H1 kinase (H1K) assay, and cytology were taken every 10 min. For immunoblot and H1K analysis, 2 µl of reaction was mixed with 20 µl of ice-chilled H1K buffer (10 mM HEPES-KOH, pH 7.7, 50 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 10 mM NaF, 5 mM EGTA), 17 µl of the mixture was added to 20 µl of 2x sample buffer [35] containing 10 mM NaF and 1 mM Na_3VO_4, boiled for 5 min and the remaining 5 µl was immediately frozen

in liquid nitrogen. For cytology samples, 2.5 µl of the reaction was mixed gently with 10 µl of EGS [ethylene glycol-bis(succinic acid *N*-hydrosysuccinimide ester)]fixative (see below). Immunoblot and H1K samples were stored at -80°C, and cytology samples were stored at 4°C.

Antibodies and immunoblotting

For immunoblot analysis [5], samples were analyzed on 12.5 to 17.5% SDS-PAGE (acrylamide/bis, 120:1) and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P filter; Millipore) at 4°C using Hoeffer immunoblotting apparatus at 100 V for 2.5 hrs or overnight at 50 mAmp. The following primary antibodies were used: affinity-purified Cdc25C antibody [5], phosphotyrosine antibody [5, 36], p90Rsk-1 antibody (product SC-231, Santa Cruz), cyclin B2 antibody [5, 37], MPM-2 antibody (Cat # 05-368, Upstate Biotechnology) and phospho-p44/42 antibody (Cat # 9101, New England Biolabs). Goat anti-rabbit or anti-mouse alkaline phosphatase-conjugated secondary antibodies (anti-rabbit; Cat. # A3687; anti-mouse; Cat. # A3562) were used at 1:3000 dilution for 1 hr at room temperature to detect the primary antibodies. Immunoblots were developed using the substrates 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) according to the manufacturer's instructions (Cat. # P3791, Promega).

Histone H1 phosphorylation assay

Histone H1 phosphorylation assays for *Xenopus* cycling egg extract reactions [5] were conducted by first rapidly thawing the H1K samples (5 µl) followed by centrifugation at 10,200xg for a few seconds at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge. Then, 3 µl of kinase mixture (20 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 0.2 mg/ml histone H1 [Boehringer Mannheim], 60 µM unlabeled ATP, 0.5 µCi/ml γ-^{32}P-ATP [NEN Life Science Products], 20 µM protein kinase A inhibitor peptide [PKI], 10 µM calmidazolium) was added to each sample, and reactions were incubated for 20 min at 21-23°C. After 20 min, reactions were quenched with 8 µl of 2x sample buffer. Samples were then separated by 15% SDS-PAGE and processed for autoradiography. For quantitation of ^{32}P incorporation, gels were analyzed by phosphorimager (Typhoon 8600).

p90Rsk-1 immunoprecipitation

Freshly made (or thawed) extracts of interphase-arrested activated eggs or M-phase-arrested unfertilized eggs were centrifuged at 10,200 x g for 5 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge. The supernatant was removed and transferred to a new 1.6-ml microfuge tube. For M-phase-arrested extracts, 1x volume of ATP-regenerating system (Roche) was added to 37x volumes of extract, and NaF was added to 20 mM final concentration. For either extract, 45 µl of p90Rsk-1 antibody was added to the final volume of 450 µl. The mixture was incubated on ice for 60 min, and transferred to a fresh tube containing 45 µl of protein A-Sepharose CL-4B beads

and Sepharose CL-4B (1:1, v/v [Amersham Pharmacia Biotech]), followed by mixing end-over-end for 1 hr at 4°C. The mixture was centrifuged at 10,200xg for 5 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge, followed by removal of the supernatant. The pelleted beads were washed end-over-end for 10 min at 4°C. They were first washed with CSF extraction buffer that contained 1.0% Tween-20, followed by wash buffer containing 1.0% Tween-20 and 1 M NaCl, then wash buffer containing 1.0% Tween-20 and twice with wash buffer. At the last stage, the pelleted beads were washed once with kinase buffer (20 mM HEPES-KOH, pH 7.0, 1 mM β-mercaptoethanol, 5 mM $MgCl_2$) and resuspended in 45 µl of kinase buffer. The resuspended immunoprecipitates were then aliquoted (10 µl slurry of 1 volume beads and 1 volume kinase buffer) and used for phosphorylation assays.

Inh-2 phosphorylation assays

The p90Rsk-1 immunoprecipitates from interphase-arrested activated egg extracts or M-phase-arrested unfertilized egg extracts were used in an *in vitro* kinase assay [26]. Aliquots were thawed and mixed with 10 µl of kinase mixture (20 mM HEPES-NaOH, pH 7.0, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mg/ml rabbit Inh-2 [Calbiochem], 60 µM unlabeled ATP, 0.5 µCi/ml γ-^{32}P-ATP [NEN]). They were incubated for 60 min at 21-22°C and quenched with 2x sample buffer. Samples were then separated by 17.5% SDS-PAGE and processed for autoradiography. To perform Inh-2 thiophosphorylation assay, 0.37-0.56 µg/µl of rabbit Inh-2 protein (Calbiochem), or human Inh-2 protein in buffer (0.1 mM EGTA, 1 mM dithiothreitol (DTT), 20 mM ammonium acetate, pH 5.0, which was brought to pH 7.0 by adding 1 M Tris solution or 50 mM Tris-HCl, pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.01% Brij-35, 50% glycerol) was incubated with active p90Rsk-1 (immunoprecipitated from M-phase-arrested unfertilized eggs) in 12 µl of kinase mixture (40 mM Tris-HCl, pH 7.5, 40 mM $MgCl_2$, 4 mM β-mercaptoethanol, 2 mM unlabeled γ-S-ATP, 2 µCi/µl γ-^{35}S-ATP [ICN Biomedicals Inc.]). The kinase reactions were mixed end-over-end for 8-25 hrs at 21-26°C. They were then centrifuged twice at 11,700xg for 10 min at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge. The supernatant was applied to a mini-column (Wizard kit; Promega) that contained 125-200 µl of 0.1 g/ml slurry of G-25 superfine Sephadex (Amersham Pharmacia Biotech) in the kinase buffer (20 mM HEPES-KOH, pH 7.0, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol). The column was centrifuged at 5,000xg for 30 sec at 4°C using a swinging bucket rotor in an Eppendorf 5417R microfuge and the eluate was collected in the receiving tube (this procedure was done twice for each supernatant). Samples from each supernatant were then mixed with 1x sample buffer, separated by 17.5% SDS-PAGE and processed for autoradiography to verify phosphorylation of Inh-2. The collected eluate was stored at -20°C until it was used for cycling *Xenopus* egg extract assays.

S6 peptide phosphorylation assays

For *in vitro* kinase reactions with S6 peptide, the p90Rsk-1 immunoprecipitates from extracts of interphase-arrested activated eggs or M-phase-arrested unfertilized eggs were used [38]. 10 μl of p90Rsk-1 immunoprecipitates were thawed and 10 μl of kinase mixture (20 mM HEPES-NaOH, pH 7.0, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mg/ml of human 40S ribosomal protein S6 peptide, amino acids 231-239 [Santa Cruz], 60 μM unlabeled ATP, 0.5 μCi/μl γ-^{32}P-ATP (NEN), 5 μM microcystin) was added to each reaction and they were incubated for 35 min at 21-22°C. The reactions were quenched with 10 μl of stop buffer (100 mM $Na_4P_2O_7$, 10 mM Na_3VO_4, 10 mM EDTA, 100 mM NaF), centrifuged, and the supernatant removed. For scintillation counting, 10 μl of supernatant was spotted onto a half-inch square of P81 (phosphocellulose) paper and dried. Each P81 square was washed 5 times with 0.5% phosphoric acid then dried. Each square was placed in a separate vial with 5 ml of scintillation liquid (Labindustries, Inc.) and counted using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, Inc.).

Autoradiography and phosphorimager analysis

For autoradiography, dried gels were placed in a cassette over X-OMAT film (Kodak) for 1 to 24 hrs. The film was developed using the Kodak X-OMAT M35 processor (Kodak). The quantitation of ^{32}P and ^{35}S incorporation was determined by phosphorimager analysis. The dried gels were placed on a phosphorimager plate for 10 to 24 hrs, processed by phosphorimager and quantified using the ImageQuant 5.1 program.

Cytology and fluorescence/phase contrast microscopy

The EGS fixative was prepared [1 volume of 0.2 M EGS dissolved in dimethyl sulfoxide (DMSO); 24 volumes of 100 mM KCl, 5 mM $MgCl_2$, 2.5% glycerol, 20 mM HEPES-KOH, pH 7.5 and 2 μg/ml Hoechst 33342 (Roche)] [5] and centrifuged at 13,000xg for 5 min at room temperature using a fixed angle rotor in an Eppendorf microfuge. The cytology samples were then examined by mounting 8.5 μl of fixed samples on a microscope slide using a wide-orifice pipette tip and covered with a 22 mm square cover slip. Samples were then examined using a Zeiss light/fluorescence microscope under 40x or 100x oil immersion objectives. Photographs were taken with a microscope camera (Zeiss-MC100; Carl Zeiss Meditec Inc.) using ELITE Chrome100 slide film (Kodak).

RESULTS

To determine whether Inh-2 protein can induce an M-phase arrest in mitotic extracts, we used *Xenopus* cycling egg extracts that mimic cell cycle events *in vitro*, as they would occur *in viv*o. The control reaction undergoes two cell cycles as indicated by entry into M phase at 50 and 100 min (Fig. 1A left panel). This is marked by an M-phase-specific transient Cdc25 hyperphosphorylation, M-phase phosphoproteins (MPM-2) phosphorylation (Fig. 1A), and M-phase nuclear morphology changes that include nuclear envelope breakdown and chromosome condensation (Fig. 1B). The addition of Inh-2 protein prior to the start of the incubation of the extracts at 21-23°C had no effect up to the start of

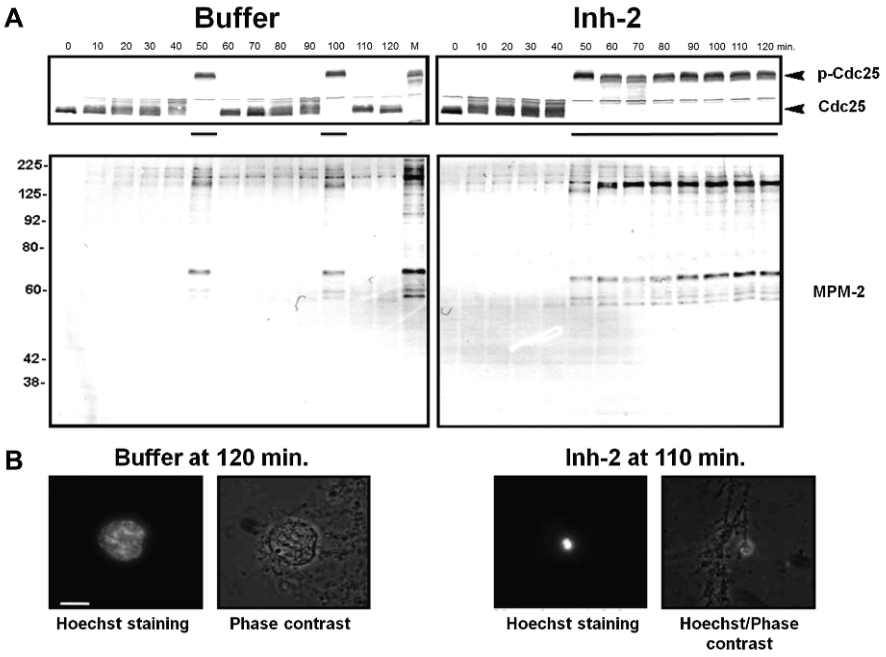

Fig. 1. Inh-2 can induce M-phase arrest in mitotic extracts. *Xenopus* cycling egg extract was prepared, and either buffer or rabbit Inh-2 protein was added to 14% (v/v) of the reaction (A). The reactions were incubated at 21-23°C and samples were taken every 10 min for analysis. Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. Top panel: Cdc25 immunoblot. Bottom panel: MPM-2 immunoblot. (M) denotes an extract of M-phase-arrested, unfertilized eggs. The p-Cdc25 arrow solid line denotes the hyperphosphorylated form of these proteins. The bars below the Cdc25 immunoblot indicate samples in which M-phase morphology of nuclei was observed (nuclear envelope breakdown [NEBD] and chromosome condensation [CC]). Cytology results of the reactions to which buffer and rabbit Inh-2 protein were added (from two different experiments) (B). The Hoechst stains chromatin; Phase contrast and combined Hoechst/ Phase contrast show the presence/absence of a nuclear envelope. Bar, 10 μM.

M phase (at 50 minutes). However, sustained hyperphosphorylation of Cdc25 and MPM-2 phosphorylation indicated that Inh-2 protein blocked cell cycle progression and induced an M-phase arrest. This was further corroborated by cytological results, in which the extracts manifested characteristic M-phase condensed chromatin with no nuclear envelope (Fig. 1B, right panel). In contrast, the control reaction had decondensed chromatin and re-formed nuclear envelope, indicating that the reaction had entered interphase (Fig. 1B, left panel).

We next determined whether the sustained mitotic protein phosphorylation induced by Inh-2 could be due to sustained activity of Cdc2 kinase, a molecule that plays a key role during M phase and whose activation is dependent upon cyclin B2 interaction. In our experiments, Cdc2 kinase activity correlated well

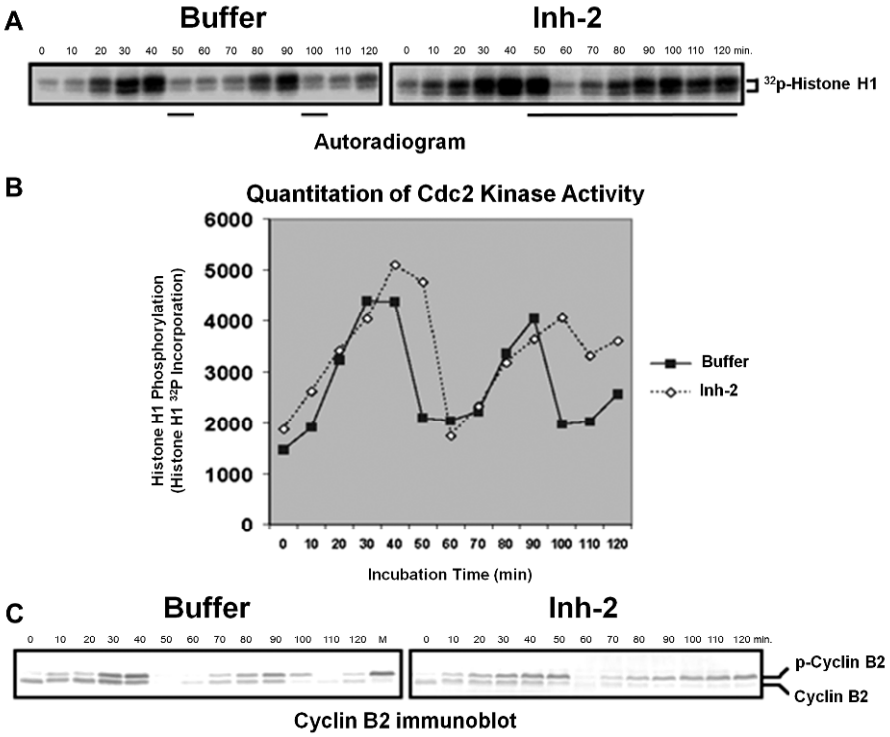

Fig. 2. Cdc2 and cyclin B activity oscillate despite cell cycle arrest. Samples taken from the experiment shown in Fig. 1(A) were assayed for Cdc2 kinase activity (A). The histone H1 kinase assay was done in presence of γ-^{32}P-ATP and histone H1 as an exogenous substrate. Kinase reactions were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Bars indicate samples in which M-phase morphology of nuclei was observed (nuclear envelope breakdown [NEBD] and chromosome condensation [CC]). Quantitation of ^{32}P incorporated into phosphorylated histone H1 by phosphorimager (B). Samples were immunoblotted with anti-cyclin B2 antibody (C). (M) denotes an extract of M-phase-arrested, unfertilized eggs.

with cyclin B protein degradation, as measured by its ability to phosphorylate the exogenous protein histone H1 *in vitro* in the presence of radiolabelled γ-^{32}P-ATP (Fig. 2A) [39]. For control samples, the Cdc2 activity decreased at 50 and 100 minutes. This coincided with diminished levels of cyclin B2 protein at 50 and 110 min (Fig. 2C, left panel). Interestingly, when Inh-2 was added to the extracts, cyclin B2 oscillation continued during the first cell cycle. However, at around 100 min (during the second cell cycle) there was a divergence between control and Inh-2 added reactions. We suspect that the increased activity of Cdc2 in the second round may be an indirect effect of the static state of cell cycle related proteins. Overall, this result suggests that cell cycle arrest could be induced by Inh-2 without sustained Cdc2 kinase activity.

The p42MAPK pathway is transiently activated during M phase of the mitotic cell cycle [3, 5, 40, 41]. We also observed the transient activation of p42MAPK in some of our immunoblots at the same time as Cdc25 phosphorylation. Similar to Cdc25 phosphoprotein in our experiments, the p42MAPK pathway activity is sustained in Inh-2 induced M-phase arrested extracts, as indicated by sustained phosphorylation of p42MAPK and p90Rsk-1(Fig. 3A). It has already been shown that mitotic activity can be arrested by p42MAPK activation after cyclin B degradation [7]. We therefore examined whether p42MAPK pathway activation plays an essential role in Inh-2-induced M-phase arrest. In order to accomplish this, we added the MEK specific inhibitor PD98059 to the reactions [42, 43] and observed that p42MAPK inactivation by PD98059 does not affect Cdc2 kinase activity or entry into M phase in *Xenopus* cycling egg extracts, but the PD98059-treated extracts show premature exit from M phase as compared to control [40]. In control experiments where only DMSO was added, the extract underwent one cell cycle as indicated by entry into M phase at 80 min, characterized by Cdc25 hyperphosphorylation, followed by its downregulation (Fig. 3B, upper panel). In this sample, p42MAPK activation was not detected by immunoblotting with anti-phospho-p44/42 antibody (Fig. 3B, lower panel). Addition of Inh-2 resulted in the entry and arrest of cell extract in M phase at 80 min (Fig. 3C, upper panel), followed by p42MAPK sustained activation (Fig. 3C, lower panel). Interestingly, inhibition of MAPK activation through PD98059 addition resulted in a normal entry and exit from M phase (Fig. 3D).

To further determine the role of the p42MAPK pathway in Inh-2 induced M-phase arrest, we examined the involvement of its downstream kinase, p90Rsk kinase, shown to be required for M-phase arrest in *Xenopus* cycling egg extracts [8]. Since p90Rsk-1 has been shown to phosphorylate rabbit Inh-2 *in vitro* [26], we immunoprecipitated p90Rsk-1 from M-phase-arrested *Xenopus* egg extracts to determine its activity (data not shown). *In vitro* kinase assays with rabbit Inh-2 protein as a substrate revealed that active p90Rsk-1 showed approximately 20-fold higher levels of P^{32} incorporation than Inh-2 phosphorylated by p90Rsk-1 immunoprecipitated from interphase extract.

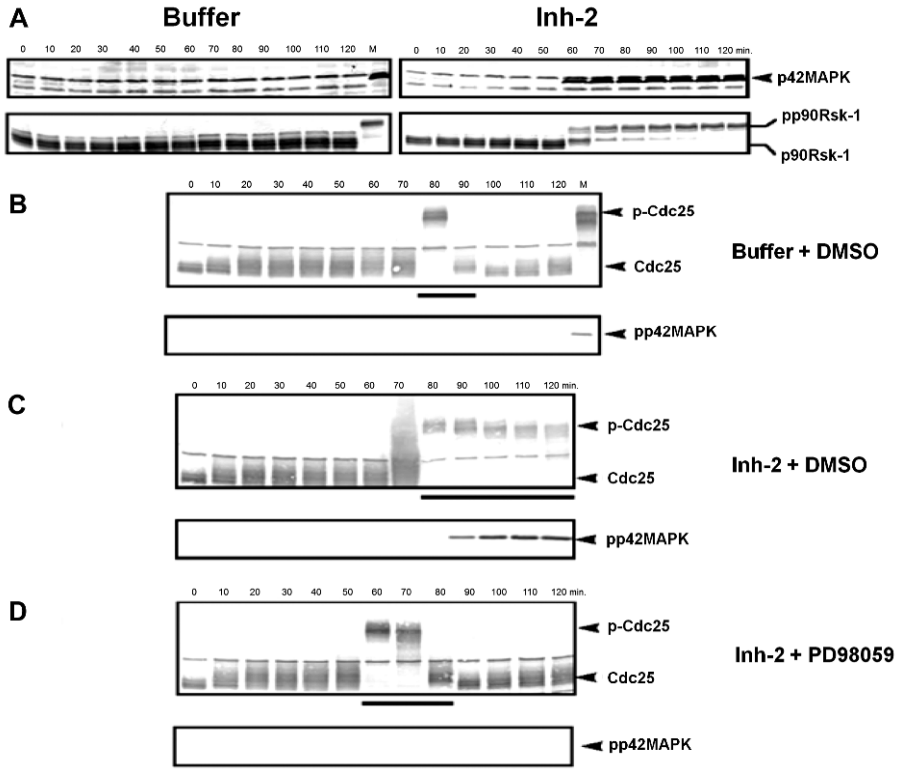

Fig. 3. Inh-2-induced M-phase arrest requires activation of the p42MAPK pathway. A *Xenopus* cycling egg extract was prepared, and either buffer or His-tagged rabbit Inh-2 protein was added to 14% for A and 5% (v/v) for B-D of the final reaction. The samples were analyzed by SDS polyacrylamide gel electrophoresis. Top panel: p42MAPK tyrosine phosphorylation (4G10) immunoblot (A). Bottom panel: p90Rsk-1 immunoblot. (M) denotes an extract of M-phase-arrested, unfertilized eggs. For B to D, either DMSO or 300 μM (final concentration) of PD98059 was added at the same volume to these reactions. Upper panel of each pair: Cdc25 immunoblot (p-Cdc25 arrow denotes the hyperphosphorylated form). Lower panels: activated p42MAPK (phospho-p44/42) immunoblot (pp42MAPK denotes the phosphorylated form). The bars below the Cdc25 immunoblots represent samples in which M-phase morphology of nuclei was observed. Reaction to which buffer and DMSO were added (B). Reaction to which Inh-2 and DMSO were added (C). Reaction to which Inh-2 and PD98059 were added (D).

To demonstrate that Inh-2 phosphorylation was not due to other M-phase kinases that were precipitated with the active p90Rsk-1, an interphase-arrested egg extract was prepared in which only p42MAPK was specifically activated. Similarly, the p90Rsk-1 immunoprecipitated from the constitutively active MEK protein [5, 44] -activated interphase-arrested extract was also able to phosphorylate Inh-2 protein (data not shown). We next examined whether p90Rsk-1-phosphorylated Inh-2 can induce an M-phase arrest similar to that induced by the un-phosphorylated form. The reaction to which thiophosphorylated

Inh-2 protein was added entered M phase at 70 min, and was arrested in M phase in a manner very similar to the unphosphorylated form (Fig. 4A). In addition, once the reaction entered M phase, thiophosphorylated Inh-2 protein underwent further post-translational modification, as observed by its slower migration in SDS-PAGE (Fig. 4B). This post-modification may be due to phosphorylation of Inh-2 by kinases other than p90Rsk-1, activated during M phase [30]. The Cdc2 kinase activity was not sustained and oscillated in thiophosphorylated Inh-2 containing reactions (data not shown). Overall, these results demonstrate that thiophosphorylation of Inh-2 by p90Rsk-1 can also induce M-phase arrest in a *Xenopus* cycling egg extract.

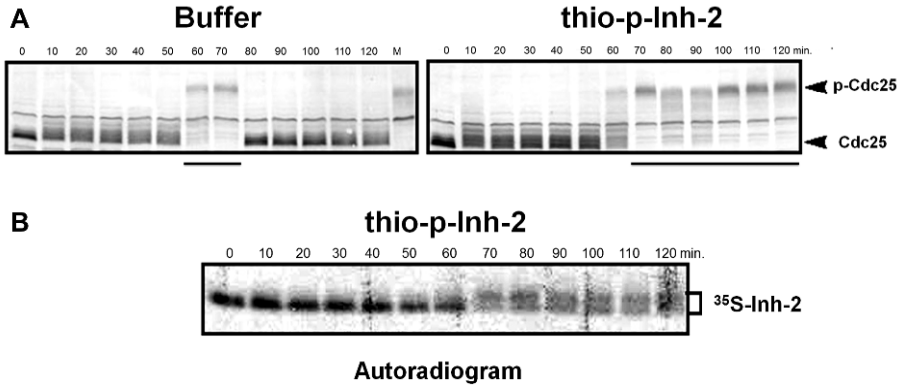

Fig. 4. p90Rsk-phosphorylated Inh-2 can induce an M-phase arrest similar to that induced by its unphosphorylated form. A *Xenopus* cycling egg extract was prepared, and either buffer or thiophosphorylated (thio-p-) rabbit Inh-2 protein was added to the final reaction. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting (A). Top panel: Cdc25 immunoblot. (M) denotes an extract of M-phase-arrested, unfertilized eggs. The bars below the Cdc25 immunoblot indicate samples in which M-phase morphology of nuclei was observed. Samples from thiophosphorylated Inh-2 reaction were separated on a 17.5% SDS-polyacrylamide gel, stained with Coomassie blue, destained and then dried (B). The autoradiogram of the gel is shown.

There could be two reasons for the similarity in potency of phosphorylated and unphosphorylated forms of Inh-2 in our experiments. First, it is possible that the concentrations used were beyond their effective threshold. By lowering the concentrations of both phosphorylated and unphosphorylated Inh-2 proteins added to *Xenopus* cycling egg extracts, we determined the effective concentration of each Inh-2 form to be between 1.6 and 2.1 μM. At concentrations of 2.1 μM, both phosphorylated and unphosphorylated Inh-2 were able to induce M-phase arrest, while at 1.6 μM they were both ineffective (Suppl. Fig. 1 in Supplementary material at http://dx.doi.org/10.2478/s11658-011-0030-z). As both p90Rsk-1 phosphorylated and unphosphorylated Inh-2 display similar potentials in their ability to induce an M-phase arrest, p90Rsk phosphorylation of Inh-2 is unlikely to be required for this M-phase arrest.

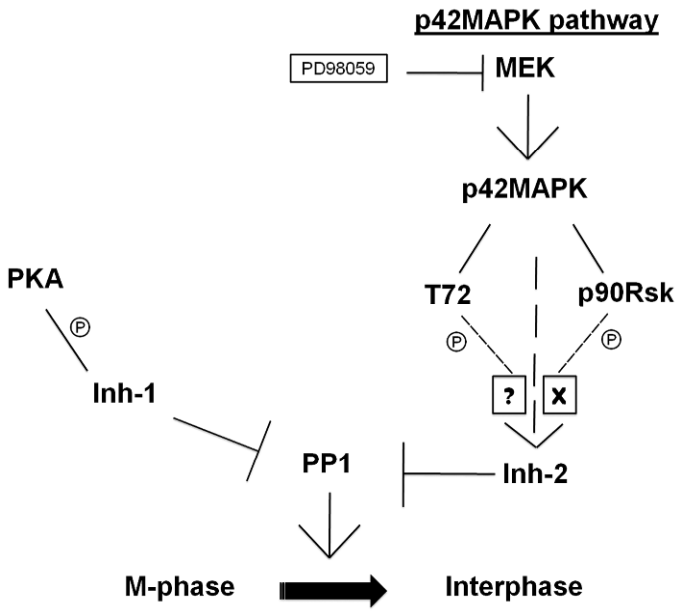

Fig. 5. Schematic model showing Inh-1 and Inh-2-induced M-phase arrests in *Xenopus* cycling egg extracts.

Alternatively, the similarity in potency could be caused by phosphorylation of the Inh-2 protein by the endogenous p90Rsk-1 subsequent to Inh-2 addition to the reaction. To circumvent this potential problem, we used human Inh-2 protein lacking the consensus site required for p90Rsk-1 phosphorylation. The p90Rsk-1 phosphorylation consensus motifs are Arg/Lys-X-Arg-X-X-Ser/Thr and Arg-Arg-X-Ser/Thr [45]. The rabbit and *Xenopus* Inh-2 amino acid sequences each have one potential p90Rsk-1 phosphorylation motif (a.a. 25-28), while human Inh-2 protein has none. Predictably, the activated p90Rsk-1 immunoprecipitated from M-phase-arrested extracts was able to phosphorylate the rabbit Inh-2 protein but failed to phosphorylate human Inh-2 protein (Suppl. Fig. 2A, B in Supplementary material at http://dx.doi.org/10.2478/s11658-011-0030-z). Despite the lack of phosphorylation, human Inh-2 protein was able to arrest the cell cycle at M phase with the same characteristics as the rabbit Inh-2 (Suppl. Fig. 2C, right panel in Supplementary material at http://dx.doi.org/10.2478/s11658-011-0030-z). Since p90Rsk-1 did not phosphorylate human Inh-2 protein *in vitro*, this result suggests that phosphorylation of the serine residue within the p90Rsk-1 consensus motif is not required for the Inh-2-induced M-phase arrest. Therefore, although Inh-2 M-phase arrest is dependent on activation of the p42MAPK pathway, Inh-2 protein does not require p90Rsk phosphorylation for the M-phase arrest. However, this result does not exclude the possibility that phosphorylation of other Ser/Thr residues present in rabbit Inh-2 (outside the consensus motif) or within the human Inh-2 sequence could contribute towards modifying Inh-2 function.

DISCUSSION

The mitotic M phase is a remarkably orchestrated process that requires PP1 phosphatase activity during completion of the latter stages. Here, we have established that a known inhibitor of PP1, Inh-2, can induce M-phase arrest in *Xenopus* cycling extracts. This induced M-phase arrest is concentration dependent and, more importantly, it requires activation of the p42MAPK pathway. The Inh-2-induced M-phase arrest is characterized by sustained M-phase nuclear morphology (chromosome condensation and nuclear envelope breakdown), as well as maintenance of M-phase specific phosphoproteins in phosphorylated or hyperphosphorylated states. This arrest also results in sustained activation of p90Rsk-1, downstream of the p42MAPK pathway. On the other hand, Cdc2 kinase activity is not sustained despite the presence of Inh-2, suggesting that not all molecular hallmarks involved in M-phase exit are affected by Inh-2 addition. This result is similar to the Inh-1 induced M-phase arrest in *Xenopus* cycling egg extracts [20] and supports previous reports that Inh-2 does not inhibit cyclin B destruction machinery or Cdc2 inactivation in these extracts [20, 32, 46, 47]. Other reports have shown that addition of Inh-2 protein causes an early entrance into M phase [32, 33], whereas in our experiments, addition of Inh-2 resulted in entry into M phase at the same time as the control and inducing an M-phase arrest. We believe that the difference with our results is due to fact that their *Xenopus* egg extracts contained a higher concentration of sperm nuclei and were therefore closer to a "somatic" cell extract with G1 and G2 phases, which retained the ability to respond to cell cycle checkpoints. Consequently, it is possible that in their experiments, the inhibition of PP1 activity affected possible checkpoint mechanisms in G1 or G2 phases, resulting in accelerated entry into M phase, whereas these mechanisms were not activated in the "embryonic" extract used in our experiments.

While the induction of an M-phase arrest by Inh-2 protein has not been shown in *Xenopus* cycling egg extracts before this report, several effects of Inh-2 on other aspects of the cell cycle involving inactivation of PP1 have been reported. Among those, Inh-2 protein was shown to inhibit DNA replication, overcome DNA checkpoint arrest, cause shortening of microtubules during anaphase, and sustain an M-phase arrest in meiotic (CSF extracts) and mitotic extracts (HeLa cells) [20, 32, 46-48]. Interestingly, Inh-1 and Inh-2 have been shown to inhibit PP1's ability to sustain phosphorylation of M-phase specific phosphoproteins after Cdc2 inactivation in meiotic and mitotic extracts [20]. The threshold concentration of Inh-2 used to induce an M-phase arrest at 2.1 μM in our extracts was just below the range reported to inhibit PP1 activity, at concentrations of 3-10 μM [32, 46, 47]. This correlates well with the notion that in our experiments, PP1 phosphatase activity is inhibited.

While Inh-1 and Inh-2 may both inhibit PP1 activity, their mode of action appears to be distinct. For Inh-1, its phosphorylation by PKA during M phase appeared to be responsible for inactivation of PP1. However, unlike Inh-1, Inh-2 does not contain the PKA phosphorylation consensus site, despite its capacity to

induce a similar M-phase arrest in *Xenopus* cycling egg extracts. Moreover, our results suggest for the first time that the Inh-2-induced M-phase arrest is a direct effect of MAPK activity, as inhibition of p42MAPK resulted in normalization of the mitotic cycle despite the presence of Inh-2 in these extracts (Fig. 5). Normally, p42MAPK becomes transiently activated, after the peak of cyclin B/Cdc2 kinase (MPF) activation in *Xenopus* cycling egg extracts [3, 5, 40, 41]. The extracts containing Inh-2 entered M phase at the same time as the control extracts, concurrent with the timing of the p42MAPK phosphorylation, meaning that the MAP kinase pathway was not prematurely activated or delayed.

The p42MAPK pathway regulates many aspects of the cell cycle in *Xenopus*, such as maturation, M-phase arrest of the unfertilized egg, and spindle assembly checkpoint [49, 50]. Interestingly, p90Rsk, which has been shown to be required downstream of the p42MAPK pathway in order to induce an M-phase or CSF arrest in *Xenopus* [8, 9], can also phosphorylate Inh-2 [26, 51]. We speculated next whether p90Rsk phosphorylation of Inh-2 might play a similar role to PKA phosphorylation of Inh-1. However, Inh-2 protein devoid of the p90Rsk phosphorylation site and p90Rsk-phosphorylated Inh-2 both had a similar threshold concentration for inducing M-phase arrests in *Xenopus* cycling egg extracts. This result suggests that p90Rsk phosphorylation of Inh-2 is not responsible for inducing or sustaining an M-phase arrest.

There is evidence linking Inh-2 and PP1 under physiological conditions, as Inh-2 protein levels increase during entry into M phase and inhibit PP1 phosphatase activity [31]. PP1 activity has been shown to oscillate during the cell cycle in *Xenopus* egg extracts with low activity during entry into M phase [32, 46, 47]. Reciprocally, a decrease in Inh-2 expression leads to increased PP1 activity during the cell cycle. The transient activation of p42MAPK observed during M phase correlates well with the PP1 inhibition and increase in Inh-2 protein levels. Although the molecular connection between Inh-2 and MAP kinase activation remains unknown, it appears that the MAPK pathway acts upstream of Inh-2, since inhibition of the p42MAPK pathway renders Inh-2 ineffective in our experiments. It may be that p42MAPK affects Inh-2 function either directly or through a different mechanism (Fig. 5). For instance, the Thr 72 residue on Inh-2 can become phosphorylated by GSK3, Cdc2 and MAPK, but the exact function of this phosphorylation remains to be investigated in *Xenopus* cycling egg extracts [23-28]. In addition, it is unlikely that activation of the MAPK pathway was induced by the spindle assembly checkpoint mechanism since proteolysis of cyclin B protein was not inhibited and our extracts contained insufficient sperm nuclei for SAC induction.

Alternatively, Inh-2 protein might also assist other kinases in inducing an M-phase arrest independently of PP1 inactivation. For instance, Inh-2 protein has been shown to interact with Pin and Aurora A kinases and increase their kinase activity *in vitro* [52, 53]. While it is possible that other kinases may also be involved, it is clear that adding exogenous Inh-2 is not sufficient to sustain M-phase arrest without MAPK activity. Further studies are required to examine their exact molecular relation during the cell cycle.

Acknowledgments. We thank Dr. Ellen Shibuya for her guidance and support in this project. We also thank Ferdinand Maingat, and Dr. Charles Holmes for their assistance with Inh-2. We would like to thank Julie Yuan for her help and support with the manuscript. This work was supported in part by grants from the Canadian Institutes of Health Research and the Alberta Heritage Fund to Dr. E. Shibuya. Arian Khandani was a recipient of a Province of Alberta Graduate Student Scholarship and a G.H. Wood award from the Faculty of Medicine and Dentistry at the University of Alberta.

REFERENCES

1. Masui, Y. and Markert, C.L. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. **J. Exp. Zool.** 177 (1971) 129-145.
2. Ferrell, J.E., Jr. Building a cellular switch: More lessons from a good egg. **Bioessays** 21 (1999) 866-870.
3. Minshull, J., Sun, H., Tonks, N.K. and Murray, A.W. A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts. **Cell** 79 (1994) 475-486.
4. Walter, S.A., Guadagno, T.M. and Ferrell, J.E., Jr. Induction of a G2-phase arrest in *Xenopus* egg extracts by activation of p42 mitogen-activated protein kinase. **Mol. Biol. Cell** 8 (1997) 2157-2169.
5. Bitangcol, J.C., Chau, A.S., Stadnick, E., Lohka, M.J., Dicken, B. and Shibuya, E.K. Activation of the p42 mitogen-activated protein kinase pathway inhibits cdc2 activation and entry into M-phase in cycling *Xenopus* egg extracts. **Mol. Biol. Cell** 9 (1998) 451-467.
6. Chau, A.S. and Shibuya, E.K. Mos-induced p42 mitogen-activated protein kinase activation stabilizes M-phase in *Xenopus* egg extracts after cyclin destruction. **Biol. Cell.** 90 (1998) 565-572.
7. Chau, A.S. and Shibuya, E.K. Inactivation of p42 mitogen-activated protein kinase is required for exit from M-phase after cyclin destruction. **J. Biol. Chem.** 274 (1999) 32085-32090.
8. Bhatt, R.R. and Ferrell, J.E., Jr. The protein kinase p90 rsk as an essential mediator of cytostatic factor activity. **Science** 286 (1999) 1362-1365.
9. Gross, S.D., Schwab, M.S., Lewellyn, A.L. and Maller, J.L. Induction of metaphase arrest in cleaving *Xenopus* embryos by the protein kinase p90Rsk. **Science** 286 (1999) 1365-1367.
10. Cohen, P.T. Protein phosphatase 1--targeted in many directions. **J. Cell Sci.** 115 (2002) 241-256.
11. Antoniw, J.F. and Cohen, P. Separation of two phosphorylase kinase phosphatases from rabbit skeletal muscle. **Eur. J. Biochem.** 68 (1976) 45-54.
12. Doonan, J.H. and Morris, N.R. The bimG gene of Aspergillus nidulans, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase 1. **Cell** 57 (1989) 987-996.
13. Ohkura, H., Adachi, Y., Kinoshita, N., Niwa, O., Toda, T. and Yanagida, M. Cold-sensitive and caffeine-supersensitive mutants of the Schizosaccharomyces

pombe dis genes implicated in sister chromatid separation during mitosis. **EMBO J.** 7 (1988) 1465-1473.
14. Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T. and Yanagida, M. The fission yeast dis2+ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. **Cell** 57 (1989) 997-1007.
15. Ishii, K., Kumada, K., Toda, T. and Yanagida, M. Requirement for PP1 phosphatase and 20S cyclosome/apc for the onset of anaphase is lessened by the dosage increase of a novel gene sds23+. **EMBO J.** 15 (1996) 6629-6640.
16. Hisamoto, N., Sugimoto, K. and Matsumoto, K. The Glc7 type 1 protein phosphatase of Saccharomyces cerevisiae is required for cell cycle progression in G2/M. **Mol. Cell. Biol.** 14 (1994) 3158-3165.
17. Vanoosthuyse, V. and Hardwick, K.G. A novel protein phosphatase 1-dependent spindle checkpoint silencing mechanism. **Curr. Biol.** 19 (2009) 1176-1181.
18. Pinsky, B.A., Nelson, C.R. and Biggins, S. Protein phosphatase 1 regulates exit from the spindle checkpoint in budding yeast. **Curr. Biol.** 19 (2009) 1182-1187.
19. Fernandez, A., Brautigan, D.L. and Lamb, N.J. Protein phosphatase type 1 in mammalian cell mitosis: Chromosomal localization and involvement in mitotic exit. **J. Cell Biol.** 116 (1992) 1421-1430.
20. Wu, J.Q., Guo, J.Y., Tang, W., Yang, C.S., Freel, C.D., Chen, C., Nairn, A.C. and Kornbluth, S. PP1-mediated dephosphorylation of phosphoproteins at mitotic exit is controlled by inhibitor-1 and PP1 phosphorylation. **Nat. Cell Biol.** 11 (2009) 644-651.
21. Eto, M., Leach, C., Tountas, N.A. and Brautigan, D.L. Phosphoprotein inhibitors of protein phosphatase-1. **Methods Enzymol.** 366 (2003) 243-260.
22. Huang, F.L. and Glinsmann, W.H. Separation and characterization of two phosphorylase phosphatase inhibitors from rabbit skeletal muscle. **Eur. J. Biochem.** 70 (1976) 419-426.
23. Hemmings, B.A., Resink, T.J. and Cohen, P. Reconstitution of a Mg-ATP-dependent protein phosphatase and its activation through a phosphorylation mechanism. **FEBS Lett.** 150 (1982) 319-324.
24. Resink, T.J., Hemmings, B.A., Tung, H.Y. and Cohen, P. Characterisation of a reconstituted Mg-ATP-dependent protein phosphatase. **Eur. J. Biochem.** 133 (1983) 455-461.
25. DePaoli-Roach, A.A. Synergistic phosphorylation and activation of ATP-Mg-dependent phosphoprotein phosphatase by F A/GSK-3 and casein kinase II (PC0.7). **J. Biol. Chem.** 259 (1984) 12144-12152.
26. Wang, Q.M., Guan, K.L., Roach, P.J. and DePaoli-Roach, A.A. Phosphorylation and activation of the ATP-Mg-dependent protein phosphatase by the mitogen-activated protein kinase. **J. Biol. Chem.** 270 (1995) 18352-18358.
27. Ballou, L.M., Brautigan, D.L. and Fischer, E.H. Subunit structure and activation of inactive phosphorylase phosphatase. **Biochemistry** 22 (1983) 3393-3399.

28. Aitken, A., Hemmings, B.A. and Hofmann, F. Identification of the residues on cyclic GMP-dependent protein kinase that are autophosphorylated in the presence of cyclic AMP and cyclic GMP. **Biochim. Biophys. Acta** 790 (1984) 219-225.
29. Li, M., Stefansson, B., Wang, W., Schaefer, E.M. and Brautigan, D.L. Phosphorylation of the Pro-X-Thr-Pro site in phosphatase inhibitor-2 by cyclin-dependent protein kinase during M-phase of the cell cycle. **Cell Signal.** 18 (2006) 1318-1326.
30. Leach, C., Shenolikar, S. and Brautigan, D.L. Phosphorylation of phosphatase inhibitor-2 at centrosomes during mitosis. **J. Biol. Chem.** 278 (2003) 26015-26020.
31. Brautigan, D.L., Sunwoo, J., Labbe, J.C., Fernandez, A. and Lamb, N.J. Cell cycle oscillation of phosphatase inhibitor-2 in rat fibroblasts coincident with p34cdc2 restriction. **Nature** 344 (1990) 74-78.
32. Walker, D.H., DePaoli-Roach, A.A. and Maller, J.L. Multiple roles for protein phosphatase 1 in regulating the *Xenopus* early embryonic cell cycle. **Mol. Biol. Cell** 3 (1992) 687-698.
33. Satinover, D.L., Brautigan, D.L. and Stukenberg, P.T. Aurora-A kinase and inhibitor-2 regulate the cyclin threshold for mitotic entry in *Xenopus* early embryonic cell cycles. **Cell Cycle** 5 (2006) 2268-2274.
34. Wang, W., Stukenberg, P.T. and Brautigan, D.L. Phosphatase inhibitor-2 balances protein phosphatase 1 and aurora B kinase for chromosome segregation and cytokinesis in human retinal epithelial cells. **Mol. Biol. Cell.** 19 (2008) 4852-4862.
35. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage t4. **Nature** 227 (1970) 680-685.
36. Druker, B.J., Mamon, H.J. and Roberts, T.M. Oncogenes, growth factors, and signal transduction. **N. Engl. J. Med.** 321 (1989) 1383-1391.
37. van der Velden, H.M. and Lohka, M.J. Mitotic arrest caused by the amino terminus of *Xenopus* cyclin B2. **Mol. Cell Biol.** 13 (1993) 1480-1488.
38. Erikson, E. and Maller, J.L. A protein kinase from *Xenopus* eggs specific for ribosomal protein S6. **Proc. Natl. Acad. Sci. USA** 82 (1985) 742-746.
39. Lohka, M.J., Hayes, M.K. and Maller, J.L. Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. **Proc. Natl. Acad. Sci. USA** 85 (1988) 3009-3013.
40. Guadagno, T.M. and Ferrell, J.E., Jr. Requirement for MAPK activation for normal mitotic progression in *Xenopus* egg extracts. **Science** 282 (1998) 1312-1315.
41. Takenaka, K., Gotoh, Y. and Nishida, E. Map kinase is required for the spindle assembly checkpoint but is dispensable for the normal M phase entry and exit in *Xenopus* egg cell cycle extracts. **J. Cell Biol.** 136 (1997) 1091-1097.
42. Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. and Saltiel, A.R. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. **J. Biol. Chem.** 270 (1995) 27489-27494.

43. Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J. and Saltiel, A.R. A synthetic inhibitor of the mitogen-activated protein kinase cascade. **Proc. Natl. Acad. Sci. USA** 92 (1995) 7686-7689.
44. Bottorff, D., Stang, S., Agellon, S. and Stone, J.C. Ras signalling is abnormal in a c-raf1 MEK1 double mutant. **Mol. Cell Biol.** 15 (1995) 5113-5122.
45. Leighton, I.A., Dalby, K.N., Caudwell, F.B., Cohen, P.T. and Cohen, P. Comparison of the specificities of p70 S6 kinase and MAPKAP kinase-1 identifies a relatively specific substrate for p70 S6 kinase: The N-terminal kinase domain of MAPKAP kinase-1 is essential for peptide phosphorylation. **FEBS Lett.** 375 (1995) 289-293.
46. Tournebize, R., Andersen, S.S., Verde, F., Doree, M., Karsenti, E. and Hyman, A.A. Distinct roles of PP1 and PP2A-like phosphatases in control of microtubule dynamics during mitosis. **EMBO J.** 16 (1997) 5537-5549.
47. Vorlaufer, E. and Peters, J.M. Regulation of the cyclin B degradation system by an inhibitor of mitotic proteolysis. **Mol. Biol. Cell** 9 (1998) 1817-1831.
48. Sohaskey, M.L. and Ferrell, J.E., Jr. Distinct, constitutively active MAPK phosphatases function in *Xenopus* oocytes: Implications for p42 MAPK regulation in vivo. **Mol. Biol. Cell** 10 (1999) 3729-3743.
49. Wu, J.Q. and Kornbluth, S. Across the meiotic divide - CSF activity in the post-Emi2/XErp1 era. **J. Cell Sci.** 121 (2008) 3509-3514.
50. Maller, J.L., Schwab, M.S., Gross, S.D., Taieb, F.E., Roberts, B.T. and Tunquist, B.J. The mechanism of CSF arrest in vertebrate oocytes. **Mol. Cell. Endocrinol.** 187 (2002) 173-178.
51. Chun, J., Chau, A.S., Maingat, F.G., Edmonds, S.D., Ostergaard, H.L. and Shibuya, E.K. Phosphorylation of Cdc25C by pp90Rsk contributes to a G2 cell cycle arrest in *Xenopus* cycling egg extracts. **Cell Cycle** 4 (2005) 148-154.
52. Li, M., Stukenberg, P.T. and Brautigan, D.L. Binding of phosphatase inhibitor-2 to prolyl isomerase Pin1 modifies specificity for mitotic phosphoproteins. **Biochemistry** 47 (2008) 292-300.
53. Satinover, D.L., Leach, C.A., Stukenberg, P.T. and Brautigan, D.L. Activation of Aurora-A kinase by protein phosphatase inhibitor-2, a bifunctional signaling protein. **Proc. Natl. Acad. Sci. USA** 101 (2004) 8625-8630.