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Regulation of MPF activation by INH/PP2A

Author
Lee, Tina H.

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Regulation of MPF Activation by INH/PP2A

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Tina H. Lee

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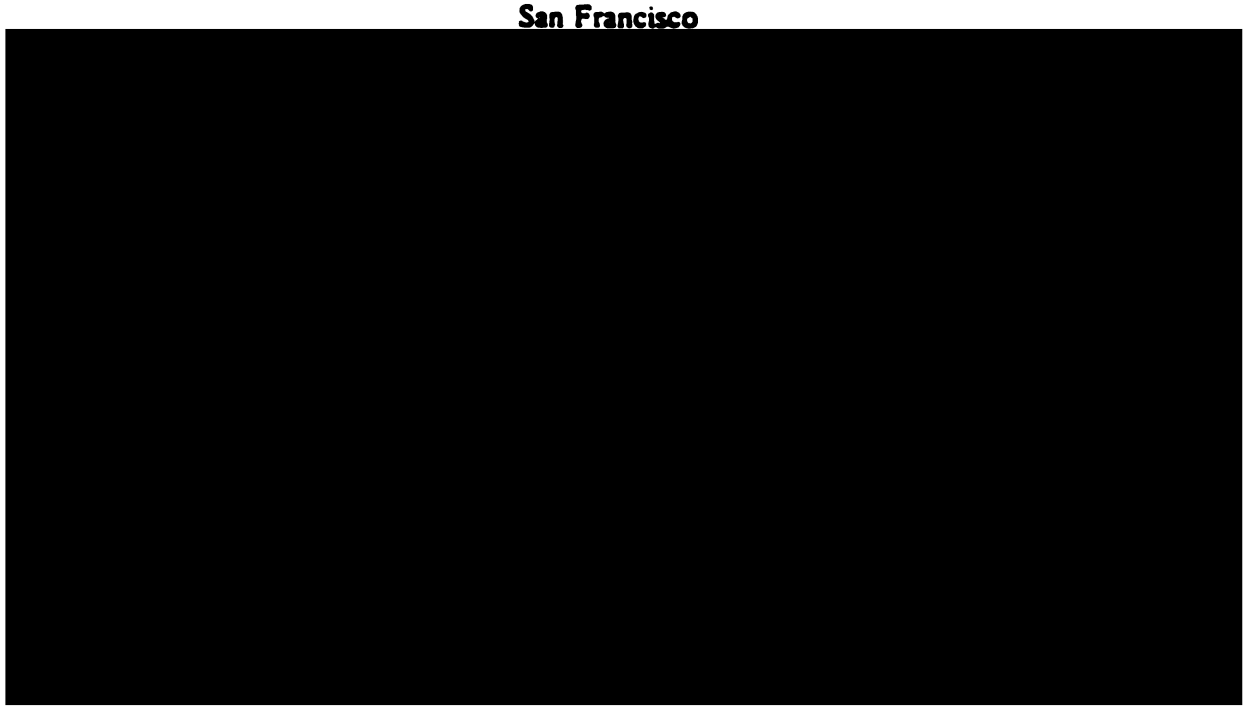

This thesis is dedicated to my parents. I thank them

for their constant support and encouragement.

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Regulation of MPF Activation by INH/PP2A

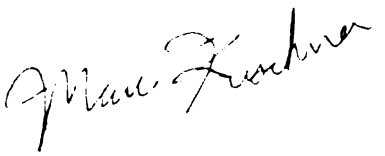
Tina H. Lee

Abstract

The abrupt appearance of M phase promoting factor (MPF) induces the G2 to M phase transition in many, if not all, eukaryotes. MPF was originally defined as a cytoplasmic factor, present in M phase cells, that could post-translationally induce M phase events when injected into G2 arrested Xenopus oocytes. M phase entry in the oocyte was accompanied by the activation of a latent form of MPF, termed preMPF. Genetic and biochemical approaches, combined, have resulted in the identification of key components of M phase induction. Cyclin B accumulation drives entry into M phase in early embryonic extracts. Initially, cyclin B associates with p34 ^{cdc2}. Following a series of post-translational modifications that function to prevent immediate activation of the complex and premature entry into M phase, the latent complex is abruptly activated as a protein kinase that is essential for maintenance of the mitotic state. The modifications on p34 ^{cdc2} that regulate the activity of the kinase complex have been extensively characterized, and many of the enzymes that modify p34 ^{cdc2} are known.

Studies in an in vitro system, designed to mimic the activation of preMPF in vivo, demonstrated the presence of an inhibitor (INH) that prevented the spontaneous conversion of preMPF to MPF in the oocyte. We purified INH and identified it as a conventional ABaC holoenzyme of type

2A protein phosphatases. INH/PP2A was shown to prevent the premature activation of the cyclin B/p34 ^{cdc2} kinase complex in egg extracts, and provided a probe for the initiating event(s). Here, we use purified INH/PP2A and Xenopus extracts to examine the mechanism by which the mitotic trigger is controlled. Our results point to the existence of unidentified components that are important for the initiation of mitosis.



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Chapter One

Introduction

Cell division is a fundamental cellular process by which the genetic material in one cell is propagated by replication and segregation into two progeny cells. Replication and segregation of chromosomes must be coordinated such that segregation does not occur until replication is complete. Thus the division process is divided into morphologically distinct phases, the DNA synthetic phase (S) and the segregation or mitotic phase (M). In most eukaryotic cells, these phases are separated by gap phases, that preceding DNA replication (G1) and that following DNA replication (G2). The G1, S and G2 phases collectively form a period known as interphase. Continuous processes of growth occur during interphase, and these processes must be coordinated with the division cycle such that a cell maintains its mass with division. While the duration of S phase and M phase is relatively constant for a given cell type, the lengths of the gap phases can vary widely, depending on environmental conditions, such as the availability of nutrients. In suboptimal growth conditions, the G1 and G2 phases are lengthened. The lengthening of these resting periods provides time for the cell to fulfill its growth requirements in preparation for the next DNA synthetic or mitotic phase. Thus there must exist control mechanisms that link growth with entry into S and M phase (Alberts et al., 1989; Murray and Hunt, 1993).

In addition to growth, there exist other controls on eukaryotic cell division. Free living unicellular organisms, such as the yeast S. cerevisiae, not only have a mechanism for delaying their division cycle when they have failed to reach a critical cell size (Hartwell and Unger, 1977), but also arrest their cell cycle in response to insufficient nutrients or upon exposure to mating pheremones (Pringle and Hartwell, 1981). Multicellular organisms face the additional challenge of coordinating the proliferation of various cell

types in different tissues according to the needs of the whole organism. In most cases, post-embryonic cell proliferation depends not only on the availability of nutrients, but also on the presence of secreted growth factors. In the absence of the necessary factors, cells arrest their division cycle and subsequently enter into a nonproliferating, quiescent state (Pardee, 1989). In contrast, early embryonic cells are equipped with a store of nutrients and sufficient stockpiles of all the structural components of the cell required to carry them through multiple cleavage divisions. With no growth requirements, these large cells undergo a rapid series of reductional divisions consisting solely of S and M phases, regardless of the extracellular environment (Graham and Morgan, 1966). Prior to the onset of these rapid cleavage divisions, however, the oocyte and egg arrest their cell cycle naturally at certain points until the occurrence of specific cues from the environment (Masui and Markert, 1971). Finally, the division of most somatic cells can be controlled by mechanisms sensing the completion of the downstream structural events of the cell cycle. For example, perturbation of DNA synthesis during S phase arrests both animal cells and yeast cells in G2 and delays entry into the next M phase, thus preventing such perturbed cells from suffering the deleterious consequences of mis-segregating their DNA (Hartwell and Weinert, 1989). In summary, cells are able to coordinate a variety of both intracellular and extracellular signals with cell division.

Studies on a wide range of cell types have revealed that for each cell type, most of the signals that affect the division cycle exert their control primarily during one cell cycle transition. Under a variety of starvation conditions, mammalian cultured cells arrest in G1, and subsequently withdraw into a quiescent state termed Go. Return to the proliferative state occurs upon exposure to the proper nutrients as well as growth factors, but

requires reentry into G1, followed by traverse across the G1/S boundary into S phase. Once cells pass the restriction point, a 'point of no return' late in G1, they are generally committed to the completion of one round of division (Pardee, 1989). An analogous 'point of no return' exists for the budding yeast, S. cerevisiae. Subthreshold cell size, lack of specific nutrients and exposure to mating pheremones all arrest the cell cycle in G1, but once cells transit a point late in G1 termed START, they are committed to that division cycle and fail to respond to environmental cues until the next G1 phase (Hartwell et al., 1974). In contrast, cell size in the fission yeast S. pombe is monitored primarily during the G2 phase, prior to entry into mitosis (Nurse, 1975). Finally, immature Xenopus oocytes are arrested for many months in a G2 like state in the ovary until the release of progesterone by the surrounding follicle cells (Maller, 1983) leads to the initiation of meiosis. In most cases, cell cycle arrest is reversible; arrested cells can be stimulated to resume the division cycle given the proper stimuli. In summary, although the point at which signals arrest progress through the cell cycle can vary from cell type to cell type, each of the transitions, Go/G1, G1/S and G2/M, can be delayed in response to specific cues. This implies the existence of specific requirements for the induction of each of these transitions.

The concept of dominance of cell cycle states surfaced from cell fusion studies in 1970 (Johnson and Rao, 1970; Rao and Johnson, 1970). Fusion of an animal cell in G1 with a cell in S phase caused the G1 nucleus to replicate precociously, suggesting the presence of a dominant factor in the S phase cell. Similarly, fusion of a mitotic cell with a cell in G1, S or G2 caused the premitotic cell to undergo nuclear envelope disassembly and premature chromosome condensation, suggesting that the mitotic state was dominant over other states. These studies coupled with studies of maturation

promoting factor in Xenopus oocytes and eggs demonstrated the existence of cytoplasmic factors that induce changes in the cell cycle state.

Immature Xenopus oocytes arrest in G2 prior to the first meiotic division. Several hours following exposure to progesterone, the oocyte undergoes meiotic maturation (entry into meiosis I, exit from meiosis I and entry into meiosis II), and finally arrests during metaphase of the second meiotic division as an unfertilized egg due to the action of cytostatic factor (CSF). In addition to progesterone, many agents can elicit the maturation response; however, several hours are generally required in a process that is protein synthesis dependent (Masui and Clarke, 1979; Huchon et al., 1981). Remarkably, Masui and Markert (1971) and Smith and Ecker (1971) discovered that the cytoplasm from the metaphase arrested unfertilized egg, when injected into the G2 arrested oocyte, could induce oocyte maturation rapidly and in the absence of any new protein synthesis. Thus it appeared that the unfertilized egg cytoplasm contained an activity that could either directly induce M phase in the recipient oocyte or activate a latent M phase inducing factor. This activity was termed maturation promoting factor (MPF). MPF activity could be detected in a wide variety of cell types (Sunkara et al., 1979), in both meiotic and mitotic cell cycles (Gerhart et al., 1984), suggesting that the mechanism for M phase induction might be highly conserved.

The generality of MPF as an M phase inducing factor was demonstrated in the Xenopus early embryonic mitotic cell cycles (Gerhart et al., 1984). Not only did MPF activity oscillate, being low in interphase and high in M phase, but MPF from the cytoplasm of unfertilized eggs could induce mitotic events when injected into embryos that had been blocked in interphase with protein synthesis inhibitors (Miake-Lye et al., 1983; Newport and Kirschner, 1984). Thus MPF seemed to bypass the protein synthesis

requirement for entry into both meiosis and mitosis, indicating the immediacy of MPF in both processes. The early embryonic cleavage cycles seemed to be driven by cycles of MPF activation and MPF inactivation.

In addition to the capacity to induce oocyte maturation in the absence of protein synthesis, the operational definition of MPF includes the characteristic of self amplification. That is, in the absence of new protein synthesis, the injection of a small amount of MPF into the oocyte triggers the activation of a latent form of MPF already present in the immature oocyte. The suggestion that MPF could activate itself was confirmed by serial transfer experiments, and led to an important conceptualization of the autocatalytic nature of MPF activation (Wasserman and Masui, 1975).

Early attempts at MPF purification were largely unsuccessful, due to the instability of the activity. However, the pioneering efforts of Gerhart and Wu (Wu and Gerhart, 1980; Gerhart et al., 1985) led to important insights into the nature of MPF. Agents that reduced the free Ca^{++} concentration and that inhibited the action of protein phosphatases were extremely important for stabilizing MPF activity. Treatment of extracts with γ-thio-ATP, an ATP analog that locks proteins in the phosphorylated state, aided the recovery of activity, and provided indirect evidence that MPF was a phosphoprotein. The involvement of phosphorylation in the regulation of MPF was not unexpected, as a burst of protein phosphorylation (Davis et al., 1983; Maller et al., 1977) and the activation of specific protein kinases (Cicirelli et al., 1988) typically accompanies entry into M phase.

Although the molecular identity of MPF was unknown, Cyert and Kirschner (1988) embarked on a study of the regulation of MPF activation that led to the identification of a potent inhibitor of the G2/M transition that is the focus of this thesis. They developed an in vitro system to study the

regulation of MPF. High speed extracts derived from immature oocytes were inefficient at generating MPF activity on their own, but generated high levels of MPF when incubated with a small amount of active MPF, mimicking the autoamplification observed in vivo. Fractionation of the crude extract revealed the existence of at least two components to the reaction, preMPF, that could be spontaneously activated by the addition of ATP, and an inhibitor of the spontaneous activation of preMPF. While removal of the inhibitor fraction (called INH) led to the spontaneous activation of preMPF, adding back the inhibitor fraction blocked the spontaneous activation of preMPF and conferred MPF dependence once again to the reaction. The activation of preMPF required ATP and the action of INH was blocked by certain inhibitors of protein phosphatases. Therefore, they postulated that INH might be a phosphatase that reversed a critical phosphorylation on preMPF that was required for its conversion to MPF.

Chapter two of this thesis describes the purification of INH and its identification as a form of type 2A protein phosphatase, a general serine/threonine phosphoprotein phosphatase (Cohen, 1989). The third chapter starts with the identification of INH as a conventional PP2A holoenzyme and goes on to describe an attempt to identify the target(s) of INH in the inhibition of MPF activation. The direction of the search was influenced heavily by the development of the cell cycle field, as will be described here.

Although MPF induced major structural changes in the cell, the regulation of MPF activity itself seemed to be independent of downstream events. Gerhart and Kirschner (1984) showed that MPF activity oscillated in eggs in the absence of DNA synthesis, spindle assembly and cytokinesis. Even enucleation of eggs had no effect on the cytoplasmic oscillator (Hara et al.,

1980). This situation seemed at odds with that in yeast and in somatic cells in general, and raised the possibility that cell cycle transitions might be controlled fundamentally differently in different organisms.

Genetic analysis of cell division cycle (cdc) mutants in the budding yeast, S. cerevisiae revealed a cell cycle consisting of a set of linear sequences of dependent events, where in contrast to the Xenopus egg, interruption of particular downstream events blocked progression to the next phase of the division cycle (Hartwell et al., 1974). In spite of the inhibitory effects of various DNA synthetic, spindle pole body duplication, etc., mutations on cell cycle progression, the yeast cell cycle exhibited the 'free running' behavior of the Xenopus embryonic cell cycle with respect to certain environmental signals. The analysis revealed a key transition point in the cycle when cell size and cues from the environment could dictate whether or not cells progressed into the next division cycle. This point, late in G1, was termed START, since it occurred prior to any known cell cycle event. However, once cells passed START, neither nutrient limitation nor mating factors could inhibit progression through the division cycle. This key transition was also defined by the mutant, $cdc28^{ts}$, that was defective in passage through START, even when all intracellular and extracellular requirements appeared to be met (Reed, 1980). $Cdc28^{+}$, therefore, represented an immediate event in the induction of passage through START, much as MPF defined an inducer of the G2/M transition.

A similar analysis of the cell cycle in fission yeast revealed that the coordination of growth with the division cycle occurred primarily at the G2/M transition (Nurse and Bisset, 1981). cdc2 mutations either arrested cells in G2 (and also at START) or accelerated entry into mitosis, revealing the product of this gene to be a critical inducer of mitosis (Nurse and Thuriaux,

1980). The interactions among the cdc2 mutants and the wee1^{ts} and cdc25^{ts} mutants revealed a network of mitotic regulators. Loss of wee1 function accelerated entry into mitosis, while the cdc25^{ts} mutant arrested cells in G2. However, the wee1^{ts} cdc25^{ts} double mutant divided normally, suggesting that cdc25^{+} and wee1^{+} antagonized one another to regulate entry into M phase (Fantes, 1979). While the wee1+ and cdc25+ gene products were dispensable in certain conditions, no other mutation could compensate for the loss of cdc2^{+}, suggesting that the cdc2 gene product was indispensable for progression through the cell cycle. Overexpression studies showed that while the amount of the cdc2^{+} gene product was not rate limiting for entry into mitosis, the cdc25^{+} and wee1^{+} gene products accelerated and inhibited, respectively, progression through the G2/M transition in a dose dependent manner (Russell and Nurse, 1986; Russell and Nurse, 1987). Sequencing of the cdc2^{+} and wee1^{+} genes indicated an homology to protein kinases. Taken together, these studies suggested that the products of cdc25^{+} and wee1^{+} opposed one another to regulate the activity of cdc2^{+}, and furthermore predicted accurately that entry into M phase would be regulated by this network of protein kinases and phosphatases.

Although the results of the studies in each of these systems were extremely informative, the generality of the mechanism of cell cycle control was not fully appreciated even with the discovery that cdc28 and cdc2 were functional homologues of one another. Complementation of the cdc2^{ts} mutant with a budding yeast library yielded the Cdc28 gene (Beach et al., 1982). Sequence comparison indicated ~65% identity of the two proteins at the amino acid level. Morever, a human homologue of cdc2^{+} was shown also to complement the cdc2^{ts} mutant, further extending the degree of conservation (Lee and Nurse, 1987). It appeared as though the cdc2^{+} gene function was

conserved in all eukaryotes. But the mechanism of action of this function was unclear at the time because no difference in the kinase activity of the cdc2/cdc28 gene products could be detected during the mitotic cell cycle (Simianis and Nurse, 1986), although it was later realized that the kinase activity did vary, when assayed using histone H1 instead of casein.

The final push that accelerated the forward movement of the cell cycle field was the biochemical purification of an MPF activity from Xenopus unfertilized eggs (Lohka et al., 1988), and the identification of the components of the purified material as Xenopus homologues of cdc2/cdc28 gene product and cyclin B (Dunphy, et al., 1988; Gautier et al., 1988; Booher et al., 1989; Draetta et al., 1989). These findings unified the previously disparate cell cycle fields into one. Cyclins were first identified as proteins in sea urchin eggs, whose pattern of accumulation, increasing during each interphase and disappearing at the end of each mitosis, made them likely candidates for a mitotic trigger (Evans et al., 1983). The definitive experiments demonstrating this point were carried out in concentrated Xenopus activated egg extracts that could undergo multiple rounds of interphase and mitosis, driven by the periodic activation and inactivation of MPF. Protein synthesis inhibitors blocked the extract in interphase, presumably due to the inability to synthesize MPF itself or an activator of MPF. The identity of the protein that had to be synthesized anew during each interphase for entry into the subsequent mitosis was previously unknown. Murray and Kirschner (1989) asked whether cyclin could fulfill the protein synthesis requirement by destroying all endogenous mRNA's in the extract (arresting the extract in interphase) and adding back a single mRNA species encoding the cyclin protein. Remarkably, when they did this, the extract underwent multiple cell cycles. Together with antisense oligonucleotide experiments by Minshull et

al. (1989), demonstrating the necessity of cyclin translation for entry into mitosis, this experiment proved that the synthesis of cyclin protein was both necessary and sufficient for the activation and as well as the subsequent inactivation of MPF. The inactivation of MPF, that is itself a product of mitotic induction, may be triggered by the degradation of cyclin protein (Murray et al., 1989), through a ubiquitin-mediated degradation pathway (Glotzer et al., 1991). Subsequent to the destruction of cyclin at the end of mitosis, the degradation machinery is turned off, once again allowing the accumulation of cyclin for the next G2/M transition.

The experiments demonstrating the sufficiency of cyclin for mitotic induction in Xenopus eggs indicated that cyclin either activated MPF by a post-translational mechanism, or was MPF itself. The relationship between cyclin and the gene product of $cdc2^+$ ($p34^{cdc2}$) was revealed soon thereafter by both genetic and biochemical approaches. Biochemical studies showed cyclin B to be stably associated with $p34^{cdc2}$ during M phase, at which time the kinase activity of $p34^{cdc2}$ toward histone H1 was dramatically higher than in interphase (Dunphy, et al., 1988; Gautier et al., 1988; Booher et al., 1989; Draetta et al., 1989); and genetic interactions were observed in S. pombe between the cdc13 gene (encoding a fission yeast homologue of cyclin B) and the cdc2 gene during the G2/M transition, that suggested a physical interaction between the two gene products in vivo (Booher et al., 1989).

Although cdc28 was considered as an inducer of the G1/S transition in budding yeast, and cdc2 was considered primarily as an inducer of the G2/M transition, extensive analyses revealed that the cdc2/cdc28 function was required for both transitions in both organisms (Nurse and Bisset, 1981; Piggott et al., 1982), suggesting a commonality to control of cell cycle transitions. In higher eukaryotes, there appears to be a family of $p34^{cdc2}$-

like (cdk) proteins involved in regulating the various cell cycle transitions (Meyerson et al., 1992), and partner to these p34 ^{cdc2}-like proteins are an ever increasing number of cyclins (Hunt, 1991). The A type cyclins associate with p34 ^{cdc2} during the G2/M transition, and the activity of the complex rises and falls with slightly differing kinetics, but their functional relationship to the mitosis-specific cyclin B/p34 ^{cdc2} complex remains unclear (Minshull et al., 1990). As compared to the cyclin B/p34 ^{cdc2} complex, less is understood about the regulation of the other cyclin/cdk complexes. It would be hoped that an understanding of the mechanisms governing control of the cyclin B/p34 ^{cdc2} complex would provide a paradigm for the regulation of cdk/cyclin complexes in general, and ultimately reveal the broader workings of cell cycle transitions.

Since the initial identification of cyclin B and p34 ^{cdc2} as components of an M phase specific histone H1 kinase important for MPF, studies carried out in a large number of laboratories, taking advantage of both genetic and biochemical techniques, has led to a general consensus on the regulation of the mitosis-specific p34 ^{cdc2}/cyclin B protein kinase complex (Solomon, 1993). In most cases, the level of the p34 ^{cdc2} polypeptide is constant throughout the cell cycle, while cyclin protein is degraded specifically at the end of each mitosis, and must be synthesized anew during each interphase. During interphase, cyclin must accumulate to a critical threshold level if it is to trigger the activation of p34 ^{cdc2}. However, accumulation of cyclin in itself is not sufficient to trigger mitosis, as there is an ~20 minute lag period between the time of cyclin accumulation and the final activation of p34 ^{cdc2} as a kinase. During the lag, p34 ^{cdc2} binds to cyclin, but the complex is inactive because it lacks an essential phosphorylation on an activating site, threonine 161. Cyclin binding allows phosphorylation on threonine 161, but

it also simultaneously induces phosphorylation on two inhibitory residues, tyrosine 15 and threonine 14. Below the threshold level of cyclin, the complex remains in the triply phosphorylated, inhibited form. Above the threshold level, cyclin catalyzes a series of events that leads finally to the activation of the phosphatase(s) that remove the inhibitory phosphorylations and to the inactivation of the kinase(s) that phosphorylates the inhibitory sites.

At least one kinase (weel) and one phosphatase (cdc25) that phosphorylates and dephosphorylates, respectively, the inhibitory sites on p34 ^{cdc2} has been identified. However, there are indications that additional enzymes with each of these activities exist (Lundgren et al., 1991; Millar et al., 1992; Kuang et al., unpublished results). Nevertheless, it is clear that the activities controlling the phosphorylation and dephosphorylation of the inhibitory sites, as a whole, are differentially regulated during the cell cycle (Solomon et al., 1990; Izumi et al., 1992; Kumagai and Dunphy, 1992; Smythe and Newport, 1992). During interphase, the inhibitory kinase(s) are highly active while the activating phosphatase(s) are largely inactive. The situation changes dramatically during the G2/M transition, when the accumulation of a threshold level of cyclin throws a switch that activates the activating phosphatase and inactivates the inhibitory kinase; these changes then lead to the sudden activation of the p34 ^{cdc2}/cyclin B protein kinase complex.

The involvement of INH in the activation of p34 ^{cdc2} was demonstrated by the use of a highly specific inhibitor (okadaic acid) of type 2A protein phosphatases in Xenopus extracts (Felix et al., 1990; Solomon et al., 1990). The effect of okadaic acid addition was two-fold. First, the p34 ^{cdc2}/cyclin B complex activated prematurely without the characteristic lag.

Second, the threshold requirement for cyclin was abrogated. Further analysis revealed an antagonistic relationship between INH and cyclin; that is, increasing the level of INH raised the threshold requirement for cyclin (Solomon et al., 1990). These results indicated that INH activity set the threshold requirement for cyclin and was essential for preventing the immediate and premature activation of p34 ^{cdc2} during interphase. INH and cyclin appeared to antagonize one another in controlling the mitotic trigger. Most importantly, the implications of these results reframed the question of the target of INH as the question of the identity of the trigger for mitosis. Chapter three of this thesis describes our attempt to approach this issue.

Chapter Two

INH, a Negative Regulator of MPF is a Form of Protein Phosphatase 2A

Summary

MPF, a protein kinase complex consisting of cyclin and $p34^{cdc2}$ subunits, promotes the G2 to M phase transition in eukaryotic cells. The pathway of activation and inactivation of MPF is not well understood, although there is strong evidence that removal of phosphate from a tyrosine residue on $p34^{cdc2}$ is part of the activation process. INH was originally identified as an activity that could inhibit the posttranslational activation of a latent form of MPF, called pre-MPF, in immature (G2 phase–arrested) Xenopus oocytes. We have purified INH and demonstrated that it is a form of protein phosphatase 2A. Both INH and the catalytic subunit of protein phosphatase 2A can directly inactivate an isolated $p34^{cdc2}$–cyclin complex. Both cyclin and $p34^{cdc2}$ become dephosphorylated; the rate of inactivation closely parallels the removal of phosphate from a specific site on $p34^{cdc2}$. We propose that INH opposes MPF activation by reversing this critical phosphorylation.

Introduction

The induction of mitosis and meiosis in eukaryotic cells is the result of a highly conserved network of biochemical reactions that culminate in the activation of a protein kinase named maturation-promoting factor (MPF). Purified MPF contains a catalytic subunit homologous to $p34^{cdc2}$ found in fission yeast (Arion et al., 1988; Dunphy et al., 1988; Gautier et al., 1988; Labbé et al., 1988; Lohka et al., 1988; Labbé et al., 1989) and a regulatory subunit called cyclin (Draetta et al., 1989) that oscillates in abundance during the cell cycle (Evans et al., 1983). During interphase, both the accumulation of newly synthesized cyclin (Minshull et al., 1989; Murray and Kirschner, 1989) and the posttranslational modification of the inactive $p34^{cdc2}$–cyclin complex (referred to as pre-MPF) are required for the activation of MPF (Booher et al., 1989; Pines and Hunter, 1989; Pondaven et al., 1990). One of these modifications, the dephosphorylation of the $p34^{cdc2}$ subunit on tyrosine, correlates with activation of MPF (Dunphy and Newport, 1989; Gautier et al., 1989; Gould and Nurse, 1989; Labbé et al., 1989; Morla et al., 1989), and the role of this posttranslational modification has been strongly supported by genetic experiments in fission yeast (Gould and Nurse, 1989).

Another posttranslational event regulates MPF activity during entry into the first meiotic division in amphibian oocytes, which occurs after a prolonged G2 phase lasting several months. Small amounts of active MPF can mobilize a large store of inactive MPF (pre-MPF) in the oocyte (Wasserman and Masui, 1975; Gerhart et al., 1984); this reaction can be reproduced in vitro (Cyert and Kirschner, 1988). Oocyte extracts generate MPF spontaneously at a very slow rate, but this rate is accelerated by the addition of small amounts of MPF. Two components of the system were identified: a 0%–33% ammonium sulfate fraction that spontaneously generated MPF (called the pre-MPF fraction), and a 45%–55% ammonium sulfate fraction that inhibited pre-MPF activation and rendered the reaction dependent on added MPF (called INH). Several phenomenological properties of INH suggested that it could be a phosphatase that would reverse a phosphorylation required for MPF activation.

In the experiments described in this paper, we purify INH and demonstrate that it is a member of the protein phosphatase 2A (PP2A) family. We show in addition that the catalytic subunit of PP2A from bovine cardiac muscle, itself, has INH activity, but that the holoenzymes lack INH activity. INH acts on an isolated cyclin–$p34^{cdc2}$ complex by both inactivating it and removing phosphorylations on cyclin that are generated during mitotic activation in vitro and removing a threonine phosphorylation of $p34^{cdc2}$ that persists during activation of the $p34^{cdc2}$–cyclin complex (Solomon et al., 1990). The kinetics of the loss of activity and dephosphorylation suggests that it is this phosphorylation on $p34^{cdc2}$ that is regulated by INH. INH not only acts in the meiotic cycle but also is responsible in part for the generation of the lag in the activation of MPF in the mitotic cycle (Solomon et al., 1990). We propose that INH acts by removing phosphate from $p34^{cdc2}$ and that it governs an important step in MPF activation.

Results

Purification of INH

INH was defined by Cyert and Kirschner (1988) as an endogenous activity that inhibited the spontaneous activation of pre-MPF in crude extracts of frog oocytes. Small amounts of INH delayed the spontaneous activation but did not diminish the final level of MPF generated. A standard assay for INH was established. INH and the pre-MPF fractions were mixed and incubated for 20 min before assaying for MPF. MPF activity was measured by microinjecting the reaction mixture into oocytes. Reactions without added INH generated a detectable level of MPF (40 U/μl; for unit definition see Wu and Gerhart, 1980) in 20 min, while those with added INH generated no detectable MPF. An endpoint dilution assay was employed, and 1 U of INH activity was defined as the amount of INH required to inhibit by 50% the amount of MPF generated after a 20 min incubation (see Experimental Procedures).

Using standard chromatographic procedures, INH ac-

Table 1. Copurification of INH Activity with MLC and H1 Phosphatase Activity and PTC-1 Immunoreactivity

Step	Total Protein (mg)	Specific INH Activity (U/mg)	Total INH Activity (U)	Specific MLC Phosphatase Activity (U/mg)	Total MLC Phosphatase Activity (U)	Specific H1 Phosphatase Activity (U/mg)	Total H1 Phosphatase Activity (U)	Specific PTC-1 Immunoreactivity (μg/mg)	Total PTC-1 Immunoreactivity (μg)
45%–55% Ammonium Sulfate	2468	7	18,490	110	271,480	14	34,552	0.09	222
50°C, 10 min	1033	14	15,480	230	237,590	28	28,924	0.14	145
DEAE–Sepharose	31.7	3000	95,328	6700	212,390	2900	91,930	4.5	143
Mono Q	0.68	12,000	8250	413,000	280,840	233,000	158,440	110	75
Hydroxyapatite	0.086	16,000	1410	533,000	45,838	373,000	32,078	117	10

The unit of INH activity is defined in the Experimental Procedures. Fold purification of INH activity has not been included because the total yield of activity increases 6-fold at the DEAE step, and thus such a figure would overestimate the actual degree of purification. See the column for specific PTC-1 immunoreactivity for a more accurate determination of fold purification of INH. Also included are the corresponding specific and total activities of MLC and H1 phosphatase and the total and specific activity of PTC-1 immunoreactive material throughout the INH purification.

tivity was enriched approximately 2000-fold to near homogeneity. Briefly, the starting material for the purification was the 45%–55% ammonium sulfate fraction from high speed oocyte extracts; Cyert and Kirschner (1988) found this fraction to contain most of the INH activity and no MPF or pre-MPF activity. This material was heated to 50°C for 10 min and centrifuged at 100,000 × g to remove protein aggregates, resulting in a 2-fold enrichment with little loss of activity. The heat step was followed by three chromatographic steps: DEAE–Sepharose, Mono Q, and hydroxyapatite (see Experimental Procedures).

Table 1 summarizes the purification. Eight percent of the total activity in the ammonium sulfate fraction was recovered from the hydroxyapatite column. A polypeptide of 32 kd and a doublet of 52 and 55 kd comigrated with INH activity (Figure 1A). INH activity (material either before or after the DEAE step) eluted from a gel filtration column at ~150 kd (data not shown).

Identification of a Phosphatase Activity Associated with Purified INH

Using H1 kinase activity as an assay for MPF, we found that INH added to a pre-MPF fraction delayed the activation of H1 kinase in the pre-MPF fraction with roughly similar, but not identical, kinetics as it delayed the activation of MPF. The difference in kinetics could be explained by the finding that INH also inhibited active H1 kinase when added at the end of the reaction (data not shown). One possibility, as suggested previously by Cyert, was that INH was a phosphatase and was acting directly on the substrate histone H1 to reverse the phosphorylation by H1 kinase. We tested active INH fractions for the ability to dephosphorylate purified H1 that had been phosphorylated by the $p34^{cdc2}$–cyclin complex. Indeed, an H1 phosphatase activity copurified with INH activity in the final steps of the purification (Figure 1C).

The extent of purification of the phosphatase activity toward histone H1 was 27,000-fold (Table 1). Histone H1 is not used as a conventional phosphatase substrate, since in addition to being a substrate, as a highly basic protein it is also a potent activator of certain phosphatases (Pelech and Cohen, 1985). Phosphorylase a (data not shown) and myosin light chain (MLC), more conventional substrates, were also substrates for the phosphatase copurifying with INH activity. MLC phosphatase activity was enriched ~5000-fold during the INH purification (Table 1). Though H1 phosphatase activities and INH activities coeluted through each stage of purification, a comparison of the yields of total INH activity with those of MLC and histone H1 phosphatase activity at each stage of purification revealed some differences (Table 1; see Discussion).

We attempted to determine the relationship of INH to known phosphatases. Cohen and colleagues have demonstrated that virtually all of the serine/threonine phosphatase activity in mammalian cell extracts can be attributed to four major types of phosphatases: types, 1, 2A, 2B, and 2C (Cohen, 1989). The catalytic subunit structure as well as the behavior of each of the types is highly conserved across species. Significantly, the INH-associated phosphatase activity was sensitive to low concentrations of okadaic acid, a very specific inhibitor of phosphatase 1 (PP1) and PP2A (Bialojan and Takai, 1988). The two types of phosphatases can be distinguished by the concentration of okadaic acid required to inhibit the activity. As shown in Table 2 (last column), INH is closer to PP2A than to PP1 in its sensitivity to okadaic acid. PP1 can be more clearly distinguished from PP2A by the use of a specific inhibitor, a 19 kd heat-stable protein called inhibitor-1 (Huang and Glinsman, 1976; Nimmo and Cohen, 1978). Whereas 300 nM inhibitor-1 completely inhibited PP1 activity toward histone H1, the same concentration of inhibitor-1 had no effect on the phosphatase activity associated with INH or PP2A (data not shown).

Immunoreactivity of a Major Polypeptide in Purified INH with a Monoclonal Antibody to the Catalytic Subunit of PP2A

PP2A has been purified from many sources (Cohen, 1989) and has been shown to be an oligomeric enzyme with a highly conserved 38 kd catalytic subunit and less well-characterized associated polypeptides of 55–70 kd. A monoclonal antibody (PTC-1) directed against the catalytic subunit of bovine cardiac PP2A (Mumby et al., 1985)

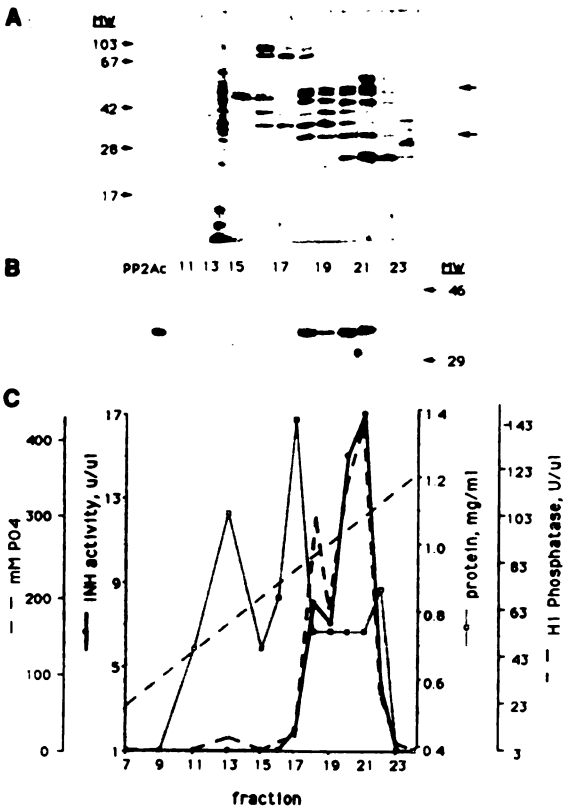

Figure 1. Fractionation on Hydroxyapatite

(A) A silver-stained 14% polyacrylamide gel showing the distribution of polypeptides across the final stage of purification. Two microliters of each fraction was loaded in each lane. Fraction numbers are indicated below and molecular weight markers (kd) are shown to the left. Fractions 18–21 comprise the peak of INH activity. The samples in (A), (B), and (C) were taken from a single set of fractions from the same column and thus can be aligned.

(B) Immunoblot across the column with a monoclonal antibody, PTC-1, directed against the catalytic subunit of bovine cardiac PP2A. Two microliters of each fraction was loaded in each lane. The positions of two of the molecular weight markers are indicated to the right, and fraction numbers are indicated above. PP2Ac indicates the lane containing 50 ng of the catalytic subunit of bovine cardiac PP2A.

(C) Column profile of INH activity and H1 phosphatase activity.

reacted with fractions from the hydroxyapatite column against a polypeptide with the same mobility as that of the catalytic subunit of bovine cardiac PP2A (Figure 1B). In addition, immunoreactivity copurified with INH in all three column steps (data shown only for the last step, Figure 1B). The extent of purification of immunoreactivity with PTC-1 is shown in Table 1. PTC-1 immunoreactivity in the hydroxyapatite fractions was purified 1300-fold from that in the 45%–55% ammonium sulfate fraction.

Phosphatase Activity and Inhibition of MPF Activation in Purified INH Show the Same Sensitivity to Okadaic Acid

The above results provide strong circumstantial evidence that INH activity, as measured by the inhibition of MPF activation, is due to the phosphatase activity in INH and that INH is a form of PP2A. The results could be explained, however, by the fortuitous copurification of PP2A with INH activity through several steps of purification. To ascertain whether INH activity was the same as PP2A activity, we examined the sensitivity of purified INH, as measured by inhibition of MPF activation, to okadaic acid and compared this sensitivity to that of the copurifying phosphatase activity, as measured on MLC. As shown in Table 3A, INH activity and phosphatase activity were inhibited by the same concentration range of okadaic acid. High concentrations of okadaic acid were required for the inhibition because the amount of catalytic subunit required for detection in the INH assay is ~100-fold greater than the amounts normally needed for the phosphatase assay, and at these concentrations of enzyme, okadaic acid appears to act stoichiometrically. Since okadaic acid alone can induce oocyte maturation (Goris et al., 1989; Pondaven et al., 1989), we had to consider the possibility that okadaic acid may be acting in this case, not by inhibiting INH activity in vitro and thereby allowing the activation of MPF, but by independently inducing maturation in the oocyte. However, we found that injection of a 7-fold higher concentration of okadaic acid was required, in our hands, to directly

Table 2. Comparison of Specific Activities of INH and Various Forms of PP2A and PP1c

Type of Phosphatase	Specific INH Activity (U/μg)	Specific H1 Phosphatase Activity (U/μg)	Specific MLC Phosphatase Activity (U/μg)	[Okadaic Acid] for 50% Inhibition of MLC Phosphatase (nM)[a]
INH (not heated)[b]	230	730	3000	8
INH (50°C)[c]	150	1270	4000	12
PP2Ac	40	400	400	6
PT-1	<1	560	438	12
PT-2	<1	790	332	12
PP1c	NA[d]	1222	144	100

Activities are defined in units per amount of catalytic subunit in the preparation; amounts of catalytic subunit were determined by immunoblotting with PTC-1 (except in the case of PP1c). PP2Ac (50% pure), PT-1, and PT-2 (60% pure) were purified from bovine cardiac tissue, and PP1c (50% pure) was purified from rabbit skeletal muscle (J. Maller).

[a] Assayed using 2 nM catalytic subunit of each phosphatase, quantitated by immunoblotting (see Experimental Procedures).

[b] A peak fraction from the DEAE column loaded with a 45%–55% ammonium sulfate fraction that has not been heated (a similar fraction that has been heated to 50°C prior to chromatography on DEAE shows a specific INH activity of 533 U/μg and a specific H1 phosphatase activity of 667 U/μg).

[c] Fraction 20 from the hydroxyapatite column (see Figure 1).

[d] Not assayed; this preparation of purified PP1c was not concentrated enough to give an estimate of the level of INH activity.

Table 3. Okadaic Acid Inhibition of INH and MLC Phosphatase Activities

A	Purified INH	
[Okadaic Acid], μM	% Total INH Activity	% Total MLC Phosphatase Activity
0.2	100	100
0.4	100	72
0.8	75	72
1.6	0	0
B	**PP2Ac**	
[Okadaic Acid], μM	% Total INH Activity	% Total MLC Phosphatase Activity
0.5	100	100
1.0	100	88
2.0	25	31
4.0	0	0

A solution of either INH (a peak fraction from the Mono Q column) or PP2Ac containing 1 U/μl INH activity (2.6 μM catalytic subunit for PP2Ac and 0.4 μM catalytic subunit for INH; catalytic subunit amounts were quantitated by immunoblotting with PTC-1, see Experimental Procedures) in EB was mixed with varying concentrations of okadaic acid (diluted into EB) and assayed either for INH activity or phosphatase activity toward MLC. Percent activities are relative to the amount of activity in the absence of okadaic acid.

induce maturation (50% inhibition at 8 μM) than was required to inhibit INH activity in vitro.

If INH is a form of PP2A, we would expect that the catalytic subunit of purified PP2A would have INH activity and that both activities would be sensitive to okadaic acid in the same concentration range. As shown in Tables 2 and 3B, PP2Ac has INH activity, and both the phosphatase activity and the ability to inhibit activation of MPF show similar inhibition by okadaic acid.

INH May Be a Special Form of PP2A

The catalytic subunit of PP2A is generally found tightly associated with other polypeptides that are less well characterized; these might confer substrate specificity or other regulatory properties on the enzyme (Imaoka et al., 1983; Mumby et al., 1987; Cohen, 1989). To compare INH, which may be a multisubunit complex, with other forms of PP2A, we examined the holoenzymes of bovine heart muscle for INH activity. We also compared INH, as a phosphatase, with the catalytic subunit of the heart muscle enzyme. We normalized the specific activities of INH and the various holoenzyme forms for the content of the catalytic subunit, which was quantitated by immunoblotting with the monoclonal antibody PTC-1, using purified bovine PP2A catalytic subunit as standard.

As shown in Table 2, the specific activity of INH as a phosphatase was ~7- to 9-fold higher than that of PP2Ac when assayed against MLC and ~2-fold higher when assayed on histone H1. Its INH activity, by the conventional assay, was ~2- to 5-fold higher than that of the catalytic subunit of PP2A. Since the catalytic subunit of INH is likely to be complexed with regulatory chains, a more meaningful comparison would be with the holoenzyme forms found in bovine heart muscle: PT1, the three subunit form, and PT2, the two subunit form (Mumby et al., 1987). As shown in Table 2, PT1 and PT2 (60% pure) have no INH activity. Both, however, exhibited phosphatase activity toward H1 and MLC.

We were concerned, despite the evidence that INH eluted from gel filtration chromatography as a 150 kd complex (data not shown), that the activity of INH could be due to free catalytic chains produced during the brief heat treatment. Heat treatment of the bovine cardiac holoenzyme forms generated very low levels of INH activity. Furthermore, purification of INH without a heat step resulted in only a slightly diminished INH and phosphatase activity (50%). Therefore, the holoenzyme forms of the heart muscle PP2A and frog oocyte INH, though sharing similar catalytic subunits and other properties, seem to be distinct enzymes.

Does INH/PP2Ac Inactivate MPF or Merely Reverse the Effects of MPF-Directed Phosphorylations?

The original assay for INH showed that it blocked the activation of MPF. This could have resulted from its acting on MPF itself or on the substrates of MPF. This ambiguity is particularly obvious in considering the activity of $p34^{cdc2}$–cyclin as an H1 kinase, since we have already seen that INH can act directly as an H1 phosphatase. As shown in Figure 2A, INH can inactivate a cyclin B–$p34^{cdc2}$ complex isolated from a mitotic extract by virtue of a glutathione transferase tag fused to the cyclin molecule (prepared as described by Solomon et al., 1990). To eliminate the possibility that residual phosphatase would persist and act directly to dephosphorylate H1 in the assay for H1 kinase, we added okadaic acid to the H1 kinase assay (see Experimental Procedures). Incubation of the partially purified $p34^{cdc2}$–cyclin with PP2Ac led to a progressive loss of H1 kinase activity that was dependent on the time of treatment with PP2Ac and independent of okadaic acid in the final assay solution. This result demonstrates that the inhibitory effect of PP2Ac was not due solely to its dephosphorylation of the H1 substrate, but was due to its action directly on the $p34^{cdc2}$–cyclin complex. Although this suggests that it acted directly on $p34^{cdc2}$ or cyclin, we cannot rule out some other copurifying activity that may be regulated by INH/PP2Ac.

Dephosphorylation of a Specific Site on $p34^{cdc2}$ Correlates with H1 Kinase Inactivation

The results of Solomon et al. (1990) show that the binding of cyclin induces the phosphorylation of three sites on $p34^{cdc2}$, one on a tyrosine and two on threonines. Activation of the $p34^{cdc2}$–cyclin complex is accompanied by the dephosphorylation of two sites and the retention of the third (threonine) site. It has been reported that cyclin is also phosphorylated during activation (Meijer et al., 1989). We have found that cyclin is unphosphorylated before activation and phosphorylated on six major sites after activation. Only one of these sites was present on the glutathione transferase fusion tag (data not shown).

We have tested whether the inactivation of $p34^{cdc2}$–cy-

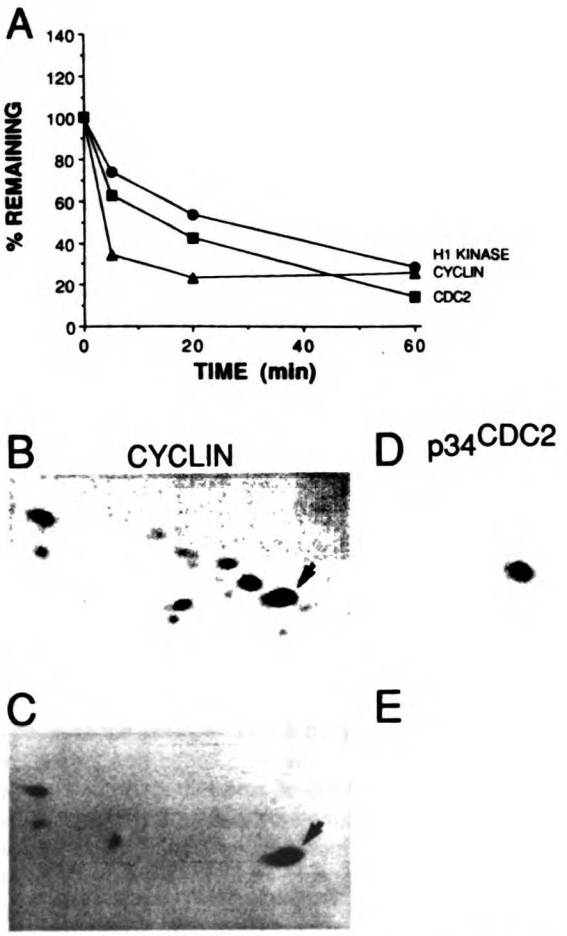

Figure 2. Inactivation of the Cyclin–p34^{cdc2} Complex by INH/PP2Ac Correlates with Dephosphorylation of p34^{cdc2}

(A) Inactivation and dephosphorylation of the cyclin–p34^{cdc2} complex by INH. An active, ^{32}P-labeled cyclin–p34^{cdc2} complex was prepared and isolated as described in Experimental Procedures and treated with 10 μl of a peak fraction from the DEAE column (3.5 μM in catalytic subunit) at 23°C. At the indicated times, the treated complex (still on glutathione beads) was washed and then eluted with glutathione. Aliquots were assayed for H1 kinase activity, electrophoresed to show cyclin phosphorylation, or immunoprecipitated with an antiserum to p34^{cdc2} prior to electrophoresis to show p34^{cdc2} phosphorylation.

(B–E) Two-dimensional tryptic phosphopeptide analysis of cyclin before (B) or after (C) treatment with INH and of p34^{cdc2} before (D) or after (E) treatment with INH. The arrows in (B) and (C) indicate a peptide derived from the glutathione transferase portion of the fusion protein. Electrophoresis was from left (anode) to right (cathode), and chromatography was from bottom to top.

clin by INH was accompanied by the dephosphorylation of cyclin and/or p34^{cdc2} and whether there was any specificity in the sites that were sensitive to INH. We isolated active, mitotically phosphorylated cyclin–p34^{cdc2} complexes (see Experimental Procedures). Both cyclin and p34^{cdc2} were dephosphorylated during H1 kinase inactivation (Figure 2A). As shown in Figure 2A, there was a rapid loss of $^{32}PO_4$ from the cyclin protein. Peptide mapping (Figures 2B and 2C) showed that this dephosphorylation occurred in parallel for all peptides except the peptide attributable to glutathione transferase. Even with this background, by 5 min 66% of the phosphate had been removed from glutathione transferase–tagged cyclin when there had only been a 26% loss of H1 kinase activity. The sites specific for cyclin were even more extensively dephosphorylated. The loss of phosphate from the single p34^{cdc2} site was more closely correlated with the loss of H1 kinase activity over the entire time course (Figures 2A, 2D, and 2E). For example, at 5 min, the H1 kinase was diminished 26% and the p34^{cdc2} phosphate 37%; at 20 min the values were 47% and 58%, respectively.

Discussion

We have purified INH activity approximately 2000-fold to near homogeneity and present evidence that it is a form of PP2A. Highly purified preparations of INH have phosphatase activity toward a variety of substrates: histone H1 phosphorylated by p34^{cdc2}, MLC phosphorylated by MLC kinase, and phosphorylase a phosphorylated by phosphorylase kinase. Phosphatase activity toward histone H1 copurified with INH activity through several chromatographic procedures. We characterized the phosphatase activity using specific inhibitors. These inhibitors indicated that the phosphatase in INH fractions was a type 2A phosphatase: The phosphatase activity was inhibited by low concentrations of okadaic acid, a specific inhibitor of PP1 and PP2A (Bialojan and Takai, 1988), but it was insensitive to inhibitor-1, a specific inhibitor of PP1 (Huang and Glinsman, 1976; Nimmo and Cohen, 1978). Reactivity against monoclonal antibody PTC-1 (Mumby et al., 1985), directed against bovine cardiac PP2Ac, copurified with INH activity through several chromatographic steps to a final increase in specific activity of about 1300-fold. Finally, PP2Ac could substitute for INH in blocking pre-MPF activation, at concentrations similar to that of the catalytic subunit present in INH fractions. Thus, INH possesses the catalytic subunit of type 2A phosphatase, and the catalytic subunit PP2Ac has INH activity. INH activity in the purified fractions was inhibited by okadaic acid in the same concentration range as was the phosphatase activity, suggesting that both are manifestations of the same enzymatic activity.

There are several reasons for thinking that INH may represent a subfraction of PP2A activities in the preparation and that the relationship between INH and PP2A could be complex. First, the holoenzyme forms from cardiac muscle were inactive in the INH assay, though they possessed phosphatase activity (Table 2). This cannot be explained by species-specific differences in the catalytic subunits, since the catalytic subunits are highly conserved among species, and the catalytic subunit prepared from the same bovine cardiac holoenzyme possessed appreciable INH activity. Second, we noticed highly reproducible anomalies in the assays for different reactions in the INH purification. In particular, we noticed a 3- to 6-fold increase in apparent total INH and H1 phosphatase activity after the DEAE–Sepharose chromatography step, without a corresponding increase in the total MLC phosphatase activity. In the Mono Q chromatographic step, there was more loss of INH activity than there was of MLC or histone phospha-

tase activity. These results can be explained by one or more of the following. There are multiple forms of PP2A that are differentially enriched at these two steps; there are specific inhibitors of INH and H1 phosphatase activity that are removed at the DEAE step but that have little effect on MLC phosphatase activity; or INH activity can interconvert between active and inactive forms during purification. A form of PP2A has been purified from Xenopus oocytes (Hermann et al., 1988); however, we do not know the relationship between that preparation and purified INH. The inability of the bovine holoenzyme to function as INH strongly suggests that the accessory chains must influence the substrate specificity or other interactions of the catalytic subunit in the INH assay. They cannot be required for these interactions, since the catalytic subunit is active in the INH assay.

The finding that INH is a form of PP2A is consistent with previous reports suggesting that protein phosphatases mediate, at least in part, the prophase block of immature Xenopus and starfish oocytes (Meijer et al., 1986; Goris et al., 1989; Pondaven et al., 1989). Although INH must be a form of PP2A, this does not tell us whether INH directly modifies components of the $p34^{cdc2}$ complex, or whether it affects the activity of $p34^{cdc2}$ indirectly. This ambiguity arises because the assay for INH activity requires a complex cell extract, and hence one cannot easily determine the substrate on which INH acts. To identify the substrate we used a more highly purified system, in which the cyclin–$p34^{cdc2}$ complex was isolated from a crude extract by virtue of a tag on the cyclin component of the complex (Solomon et al., 1990). Although this purification greatly simplifies the system, we do not know what other activities may copurify, especially at low stoichiometry. Nevertheless, in this partially purified system, we have shown that PP2Ac or INH is capable of inactivating $p34^{cdc2}$, whose H1 kinase activity can be subsequently assayed in the absence of residual phosphatase activity. This indicates that dephosphorylation of the $p34^{cdc2}$ complex inactivates MPF. This result suggests that phosphorylation of the complex is required for maintaining activity.

Recent work by Solomon et al. (1990) and this report have examined the role of INH in controlling the key regulatory reactions of the cell cycle involving cyclin and $p34^{cdc2}$. INH seems to play an important role in the mitotic as well as the meiotic cycle. Solomon et al. (1990) have demonstrated that alterations in the INH levels can change the threshold concentration of cyclin required for activation of MPF in interphase frog egg extracts. Addition of okadaic acid to interphase Xenopus egg extracts lowers the requirement for the amount of cyclin that must be added in order to activate the $p34^{cdc2}$ complex (Félix et al., 1990; Solomon et al., 1990); addition of partially purified INH raises the threshold. The response is linear; the more INH added, the greater the amount of cyclin required. The concentration of INH in the extracts accounts for the lag phase in the absence of added INH (Solomon et al., 1990). The need to overcome the inhibitory effect of INH might explain, at least in part, why the accumulation of cyclin protein is gradual throughout interphase, whereas $p34^{cdc2}$ activation is abrupt and occurs only when the amount of MPF activity that can be generated is sufficient to carry out all mitotic events (see Solomon et al., 1990).

We attempted to determine which component(s) of the $p34^{cdc2}$–cyclin complex was dephosphorylated during the inactivation reaction, and in particular, which phosphorylation site is crucial for controlling the activity of MPF. Phosphorylation of cyclin has been shown to correlate with the activation of the kinase (Meijer et al., 1989), and a single site of phosphorylation remains on $p34^{cdc2}$ after the activation of the complex (Solomon et al., 1990). In this paper we have demonstrated that INH dephosphorylates sites on both cyclin and $p34^{cdc2}$ during the course of the inactivation of the complex. The kinetics of $p34^{cdc2}$ dephosphorylation appear similar to the inactivation of H1 kinase activity, whereas the cyclin dephosphorylation occurs much more rapidly than H1 kinase inactivation. These experiments suggest that cyclin phosphorylations do not regulate activity and that the mitotic $p34^{cdc2}$ site is crucial for controlling MPF activity. Elimination of each phosphorylation site on cyclin and on $p34^{cdc2}$ will be necessary to further define these reactions.

The purification and characterization of INH suggest that phosphorylations, in addition to dephosphorylations, are required steps for the activation reaction in meiosis and mitosis. Complementary to our findings, recent work in fission yeast suggests a role for phosphatase type 2A in regulating entry into mitosis in vivo (Kinoshita et al., 1990). The existence of offsetting reactions to control a single pathway is not unusual, but it suggests the need for careful regulation. It is now clear that the lag, or G2 phase, is dependent on INH activity. Recent evidence that the length of G2 is controlled by feedback mechanisms sensitive to the completion of DNA replication and repair of DNA damage (Hartwell and Weinert, 1989; Dasso and Newport, 1990) underscores the potential importance of INH and the reactions it controls.

Experimental Procedures

Xenopus

Adult Xenopus laevis females and males were obtained from the laboratory of Dr. J. Gerhart (University of California, Berkeley). Frog care and oocyte procedures were as described in Cyert and Kirschner (1988).

Pre-MPF and INH Assay

The pre-MPF stock was prepared by making a 0%–33% ammonium sulfate fraction from high speed oocyte extracts as described previously (Cyert and Kirschner, 1988). Ammonium sulfate pellets were resuspended in EB (80 mM β-glycerol phosphate, 20 mM EGTA, 15 mM $MgCl_2$), a buffer developed to stabilize MPF activity (Wu and Gerhart, 1980), to give ~20 mg/ml protein and dialyzed against EB and frozen in aliquots at −80°C. To assay for INH, pre-MPF aliquots were thawed just prior to use and incubated with an equal volume of EB or a sample to be assayed for INH and 1/5th volume of an ATP-regenerating system (Cyert and Kirschner, 1988) for 20 min at 23°C. When the incubation was performed with added EB, it resulted in the generation of 40 U/μl of MPF. After the incubation, 50 nl of the mixture were injected into each of a group of four oocytes. The oocytes were fixed with 10% trichloroacetic acid 2 hr after injection, and dissected to determine whether or not germinal vesicle breakdown had occurred. Samples containing no INH activity resulted in 100% germinal vesicle breakdown, whereas samples containing 1 U/μl INH activity resulted in 50% germinal vesicle breakdown (2/4 oocytes). INH at ≥2 U/μl resulted in

no germinal vesicle breakdown. Serial dilutions into EB were performed to determine the amount of activity present.

Purification of INH

Whole ovaries (900 ml) from 60 adult Xenopus laevis females were washed extensively in 100 mM NaCl, followed by two washes in EB and one wash in EB, 10 mM dithiothreitol, 1× protease inhibitors (25 μg/ml leupeptin and aprotinin, 1 mM benzamidine HCl, 10 μg/ml pepstatin, 0.5 mM PMSF). The ovaries were homogenized in 1/4th vol EB, 10 mM DTT, 1× protease inhibitors, in a Waring blender (on "blend" for 5 s). The homogenate was centrifuged in a 45 Ti rotor at 150,000 × g at 4°C for 3 hr. The cytoplasmic layer was removed, and saturated ammonium sulfate in EB was added to 45%. After incubation on ice for 30 min, the precipitate was spun at 10,000 rpm at 4°C for 30 min. The supernatant was adjusted to 55% ammonium sulfate, and the resulting precipitate was collected by centrifugation at 10,000 rpm at 4°C for 30 min and resuspended in a volume of EB, 10 mM DTT, 1× protease inhibitors to give a protein concentration of ~40 mg/ml. Resuspended pellets were dialyzed against EB, 1 mM DTT, 0.1× protease inhibitors and the dialysate was heated to 50°C for 10 min and centrifuged at 150,000 × g at 4°C for 30 min. The pellet was discarded and the supernatant, which contained half of the protein and virtually all of the activity, was dialyzed against buffer A (20 mM Tris–HCl, 7 mM EGTA, 5 mM $MgCl_2$, 0.2 mM EDTA, 50 mM NaCl [pH 8.0], 1 mM DTT, 0.1× protease inhibitors. The dialysate was loaded onto a 75 ml DEAE–Sepharose CL-6B column equilibrated in buffer A, 10 mM DTT, 1× protease inhibitors. After washing the column, proteins were eluted with a 500 ml linear 50–600 mM NaCl gradient. Fractions were collected, measured for conductivity, and precipitated by adding solid ammonium sulfate to give 80% saturation. Precipitates were collected by centrifuging at 10,000 rpm at 4°C for 30 min. Pellets were resuspended in EB, 10 mM DTT, 1× protease inhibitors, and frozen at −80°C. A small portion of each fraction was dialyzed against EB, 1 mM DTT, 0.1× protease inhibitors and assayed for INH activity. Active fractions from two runs, eluting off the DEAE column at 200–250 mM NaCl, away from the majority of proteins, were pooled and dialyzed against buffer A, 1 mM DTT, 0.1× protease inhibitors. The dialysate was loaded onto a FPLC Mono Q Pharmacia HR 10/10 column equilibrated in buffer A. After washing the column, proteins were eluted with a 160 ml linear 50–500 mM NaCl gradient. Fractions were made 10 mM in DTT, 1× in protease inhibitors, and concentrated by centrifuging through Centricon-30 cells (Amicon) at 4°C for 1 hr, then dialyzed against EB, 1 mM DTT, 0.1× protease inhibitors for assay. INH activity eluted at ~250–280 mM NaCl. Active fractions from four Mono Q runs were pooled and dialyzed against buffer B (25 mM sodium phosphate, 50 mM NaCl [pH 7.0], 1 mM DTT, 0.1× protease inhibitors. The dialysate was loaded onto an HPLC hydroxyapatite TSK gel HA-1000 column equilibrated in buffer B. The column was washed and proteins were eluted with a 20 ml linear 25–350 mM sodium phosphate gradient. Fractions were concentrated as described for the Mono Q column, dialyzed against EB, 10% glycerol, 1 mM DTT, 0.1× protease inhibitors, and assayed for activity. INH activity eluted at ~250–310 mM phosphate. Fractions were stored at −80°C. Protein concentrations were determined by the Bradford assay using bovine serum albumin as standard.

Preparation of ^{32}P-Labeled Histone H1

Histone H1 was phosphorylated by a preparation of H1 kinase immobilized on agarose beads as follows. A bacterial fusion protein containing an in-frame insertion of the IgG-binding domain of protein A into a derivative of the sea urchin B-type cyclin, was purified from Escherichia coli on IgG–agarose beads (Solomon et al., 1990). The cyclin protein bound to beads was added to an equal volume of interphase extract prepared by treating a cytostatic factor–arrested extract (Murray et al., 1989) with 0.4 mM Ca^{2+} and 100 μg/ml cycloheximide and incubating at 23°C for 45 min. After 20 min at 23°C, by which time the extract had been induced to enter M phase, the cyclin and proteins bound to it ($p34^{cdc2}$) were spun out of the extract and shown to possess H1 kinase activity. Ten microliters of the H1 kinase on beads was used to phosphorylate 250 μg of histone H1 in a 60 μl reaction containing 0.4 mM ATP and 0.25 mCi/ml [α-^{32}P]ATP. After 30 min at 23°C, the supernatant was removed and chromatographed over a Sephadex G-25 desalting column. The remaining free ATP was removed by centrifugation through Centricon-30 cells. After phosphorylation, the preparation contained 0.2 mol of phosphate per mol of H1.

Phosphatase Assays

For H1 phosphatase assays 1 μl of diluted phosphatase and 1 μl of phosphorylated histone H1 were added to 8 μl of TE (20 mM Tris–HCl, 1 mM EDTA, [pH 7.4]) to give a final substrate concentration of 2 μM. After 5 min at 23°C, 20 μl of 50% TCA and 20 μl of 6 mg/ml bovine serum albumin were added, and the mixture was incubated on ice for 5 min. TCA-insoluble counts were precipitated by centrifuging in a microfuge for 2 min. Forty-five microliters of the supernatant was added to 2 ml of Equolome scintillation fluid and counted. One unit represents release of 0.02 pmol of phosphate per min at room temperature.

For MLC phosphatase assays, phosphorylated bovine cardiac myosin light chains were prepared as described (Mumby et al., 1985). After phosphorylation, the preparation contained 1.0 mol of phosphate per mol of MLC. Assays were carried out in 10 μl reaction volumes. One microliter of diluted substrate and 1 μl of diluted phosphatase were added to 8 μl of 50 mM Tris–HCl, 0.1 mM EDTA, 0.5 mM DTT (pH 7.2) to give a final substrate concentration of 4 μM. After incubation for 5 min at 23°C, 20 μl of 50% TCA and 20 μl of 6 mg/ml bovine serum albumin were added to each reaction and incubated on ice for 5 min. TCA-insoluble counts were precipitated by spinning in a microfuge for 2 min, and 45 μl of the supernatant was added to 2 ml of scintillation fluid and counted. One unit represents the release of 1 pmol of phosphate per min. PP1c and inhibitor-1, purified from rabbit skeletal muscle, were gifts from J. Maller. The okadaic acid was obtained from Moana Bioproducts Inc. in 100% DMF; after drying down, the okadaic acid was redissolved in 100% DMSO at a concentration of 124 μM and stored at −20°C.

Preparation of $p34^{cdc2}$–Cyclin B Complex

The glutathione transferase–tagged cyclin fusion protein carries glutathione transferase fused to the amino terminus of the Δ13 derivative of the sea urchin B-type cyclin (Solomon et al., 1990). The construct was expressed in E. coli and partially purified from cell lysates by virtue of the affinity of the glutathione transferase portion to glutathione–agarose beads (Solomon et al., 1990). The resulting preparation contained 200 μg/ml soluble cyclin. A 1:10 dilution of glutathione transferase–tagged cyclin fusion protein into XB (100 mM KCl, 50 mM sucrose, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM K-HEPES [pH 7.4]) was added to an equal volume of interphase extract as described above and incubated for 20 min at 23°C to generate maximal H1 kinase activity. At this time, 10 μl of the reaction mix was diluted to 0.5 ml in XB and bound to 20 μl of glutathione–agarose beads by rotating for 30 min at 23°C. After binding, the beads were washed ten times in 1 ml of XB, 0.5 M NaCl followed by ten 1 ml washes in XB. The resulting complex was shown to contain $p34^{cdc2}$ and to retain high H1 kinase activity (Solomon et al., 1990).

Inactivation of $p34^{cdc2}$ and Dephosphorylation of Cyclin by INH/PP2A

^{32}P-labeled, active cyclin–$p34^{cdc2}$ complexes were prepared as described in Solomon et al. (1990). Briefly, 25 mCi of [^{32}P]orthophosphate was added to 60 μl of interphase extract, which was subsequently added to an equal volume of glutathione beads prebound with glutathione transferase–tagged cyclin fusion protein (1:10 dilution). After a 30 min activation at 23°C, the beads were pelleted and washed extensively in XB containing 0.5 M NaCl and 0.5% NP-40, in XB, and finally resuspended in XB and aliquoted for INH treatment (10 μl of beads per aliquot). Ten microliters of XB or of a peak fraction from the DEAE column (3.5 μM in catalytic subunit) was added to each pellet and incubated at 23°C. At appropriate times, cold XB was added, and the beads were rinsed twice. Complexes were eluted in 50 μl of EB, 5 mM glutathione, 0.1 mg/ml ovalbumin, 10% glycerol, 0.1% NP-40, 1 μM okadaic acid. A 1:10 dilution was assayed for H1 kinase as described (Murray and Kirschner, 1989) but using five times the usual specific activity of [α-^{32}P]ATP. An aliquot was added to sample buffer and electrophoresed to show phosphorylated cyclin. The remainder was immunoprecipitated with a "PSTAIR" serum to $p34^{cdc2}$ as described (Solomon et al., 1990) prior to electrophoresis. Bands of H1, cyclin, and $p34^{cdc2}$ were excised and Cerenkov counted to determine

residual activity or phosphorylation. Tryptic phosphopeptide analysis was performed as described (Solomon et al., 1990; Ward and Kirschner, 1990).

Western Blots

The catalytic subunit of PP2A was detected using monoclonal antibody PTC-1 (Mumby et al., 1985), directed against the catalytic subunit of bovine cardiac PP2A. Nitrocellulose sheets were incubated in 50 mM Tris–HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM NaCl, 5% nonfat dry milk, 0.2% Tween, 0.02% sodium azide for 1 hr at room temperature with shaking. The sheets were then transferred to 30 ml of primary buffer (50 mM Tris–HCl (pH 8), 2 mM $CaCl_2$, 80 mM NaCl, 5% nonfat dry milk, 1% Tween, 0.2% SDS, 0.02% azide) containing 2-4 μg/ml PTC-1 and incubated for 1 hr at room temperature with vigorous shaking. After four 10 min washes in the primary buffer, the sheets were incubated in primary buffer containing ^{125}I-labeled goat anti-mouse IgG (100,000 cpm per lane) for 1 hr at room temperature with vigorous shaking. The sheets were washed four times for 10 min in primary buffer, followed by two 10 min washes in 50 mM Tris–HCl (pH 8), 2 mM $CaCl_2$, 80 mM NaCl. After autoradiography, bands were quantitated by scanning with a laser scanner. Purified preparations of the catalytic subunit were used as standards.

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Chapter Three

Inhibition of p34 cdc2 Activation by INH/PP2A

ABSTRACT

INH, a type 2A protein phosphatase (PP2A), negatively regulates entry into M phase and the cyclin B-dependent activation of cdc2 in *Xenopus* extracts. INH appears to be central to the mechanism of the trigger for mitotic initiation, as it prevents the premature activation of cdc2. We first show that INH is a conventional form of PP2A with a Bα regulatory subunit. We next explore the mechanism by which it inhibits cdc2 activation by examining the effect of purified PP2A on the reaction pathways controlling cdc2 activity. Our results suggest that although PP2A inhibits the switch in tyrosine kinase and tyrosine phosphatase activities accompanying mitosis, this switch is a consequence of the inhibition of some other rate-limiting event. In the preactivation phase, PP2A inhibits the pathway leading to T161 phosphorylation, suggesting that this activity may be one of the rate-limiting events for transition. However, our results also suggest that the accumulation of active cdc2/cyclin complexes during the lag is only one of the events required for triggering entry into mitosis.

INTRODUCTION

Entry into M phase in eukaryotic cells depends upon a cytoplasmic factor called MPF (maturation promoting factor) (Miake-Lye *et al.*, 1983; Gerhart and Kirschner, 1984). This activity was originally defined by its ability to induce M phase events when injected into G2 arrested immature *Xenopus* oocytes (Masui and Markert, 1971; Smith and Ecker, 1971). MPF was unique among other inducers of oocyte maturation in its ability to rapidly induce maturation in the absence of new protein synthesis. In addition, upon the induction of meiosis, much more MPF could be extracted from the oocyte than the amount initially injected, suggesting that the injected MPF had activated a latent pool of MPF, which was termed preMPF (Wasserman and Masui, 1975; Cyert and Kirschner, 1988). MPF was later demonstrated to be a protein kinase complex composed of p34 ^{cdc2} and cyclin B (Evans *et al.*, 1983; Arion *et al.*, 1988; Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Lohka *et al.*, 1988; Booher *et al.*, 1989; Gautier *et al.*, 1990), and preMPF was shown to correspond to a pool of cyclin/cdc2 complexes phosphorylated on sites that inhibit its activity (Dunphy and Newport, 1989; Gautier *et al.*, 1989).

In order to study the mechanism of this post-translational activation of MPF, Cyert and Kirschner (1988) developed an *in vitro* system that mimicked the amplification observed *in vivo.* They demonstrated that a small amount of active MPF can activate preMPF in the oocyte extract. However, removal of an inhibitor fraction from oocyte extracts allowed the activation of preMPF even in the absence of added MPF. Adding back this inhibitor fraction to preMPF blocked the spontaneous activation of preMPF, demonstrating the presence of a critical factor in the oocyte that prevented the

activation of MPF and thus locked the oocyte in interphase. This putative inhibitor was called INH.

We purified INH and identified it as a type 2A protein phosphatase, PP2A, (Lee *et al.*, 1991), one of the four major classes of serine-threonine protein phosphatases (reviewed in Cohen, 1989). Okadaic acid, a potent and highly specific inhibitor of PP2A (Bialojan and Takai, 1988), induces premature entry into both mitosis and meiosis when added to interphase cells or extracts (Goris *et al.*, 1989; Felix *et al.*, 1990), supporting the hypothesis that INH/PP2A is a key negative regulator of M phase induction. However, because PP2A has a broad substrate specificity *in vitro*, the target of PP2A crucial for M phase regulation has not yet been identified. Since the cyclin B/p34 ^{cdc2} protein kinase complex is the key regulator of M phase, PP2A must directly or indirectly regulate its activity. Consistent with this hypothesis, PP2A was shown to delay the activation of cdc2 after the addition of cyclin B to Xenopus extracts (Solomon *et al.*, 1990).

Several key features of the activation of cdc2 in interphase *Xenopus* extracts have been elucidated biochemically. Unlike oocyte extracts, which contain a stockpile of cyclin B in a complex with p34 ^{cdc2} (Dunphy and Newport, 1989; Gautier *et al.*, 1989), interphase extracts from activated eggs contain monomeric p34 ^{cdc2} and are devoid of cyclin B unless protein synthesis is allowed to occur (Minshull *et al.*, 1989; Murray *et al.*, 1989). Addition of a threshold level of cyclin B protein is both necessary and sufficient to drive the extract into mitosis, providing a powerful *in vitro* system to study the biochemical events involved in the activation of cdc2 (Minshull *et al.*, 1989; Murray *et al.*, 1989).

Solomon *et al.* found that there is an ~20 minute lag between cyclin accumulation and p34 ^{cdc2} activation (Solomon *et al.*, 1990). During the

lag, p34 ^{cdc2} binds to cyclin. This event is a prerequisite for further post-translational modifications on two independent sets of phosphorylation sites. One is an activating phosphorylation on threonine 161, catalyzed by CAK kinase (Solomon *et al.*, 1992; Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993), that is absolutely required for the kinase activity of p34 ^{cdc2} (Booher and Beach, 1986; Gould *et al.*, 1991). The other is a pair of inhibitory phosphorylations on threonine 14 and tyrosine 15 (Labbe *et al.*, 1988; Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989). In fission yeast, the tyrosine 15 phosphorylation is controlled by cdc25 (reviewed in Millar and Russell, 1992) and wee1/mik1 (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren *et al.*, 1991). cdc25 dephosphorylates both of the inhibitory sites (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Strausfield *et al.*, 1991) while wee1 appears to phosphorylate only tyrosine 15 (Parker and Piwnica-Worms, 1992; Booher et al., 1993; McGowan and Russell, 1993). Therefore, there may be a kinase distinct from wee1 in Xenopus extracts which phosphorylates threonine 14, although the identity of the kinase is not known (though it could be mik1). During interphase, the tyrosine kinase activity (wee1/mik1) is high and the phosphatase activity (cdc25) is low (Solomon *et al.*, 1990; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Smythe and Newport, 1992), allowing p34 ^{cdc2} to accumulate predominantly in the tyrosine phosphorylated, inactive state. During the lag, some process opposing the negative regulation by PP2A triggers the activation of the tyrosine phosphatase and the inactivation of the tyrosine kinase. These changes lead to the sudden dephosphorylation of p34 ^{cdc2} on the inhibitory sites, resulting in its abrupt conversion to the mitotic form, which is unphosphorylated on threonine 14 and tyrosine 15 but retains threonine 161 phosphorylation

(Solomon et al, 1990).

Addition of okadaic acid to the interphase extract drastically alters the kinetics of cdc2 activation by cyclin (Felix *et al.*, 1990; Solomon *et al.*, 1990). In the presence of okadaic acid, cdc2 activation occurs at subthreshold levels of cyclin, without a lag phase. Conversely, increasing the level of PP2A in the interphase extract delays cdc2 activation and raises the threshold for cyclin (Solomon *et al.*, 1990). These observations demonstrate the importance of PP2A in allowing cdc2 kinase to accumulate in an inactive state until a threshold level of cyclin has accumulated. What remains to be elucidated is how PP2A prevents the immediate and premature activation of cdc2 and how cyclin overcomes the negative regulation by PP2A.

One possibility is that cyclin and PP2A antagonize one another during the lag to control the activities of cdc25 and wee1, which cooperate to keep the cyclin B/p34 ^{cdc2} complex inactive. PP2A might prevent the activation of cdc25 and the inactivation of wee1, whereas cyclin would promote these processes. A threshold level of cyclin would normally be required to overcome the inhibition by PP2A. However, in the absence of PP2A activity, low levels of cyclin could suffice to turn on cdc25 and turn off wee 1 relatively quickly. Studies on the regulation of cdc25 and wee 1 have, in fact, pointed to an essential role for PP2A in maintaining the low level of cdc25 activity as well as the high level of wee1/mik1 activity. According to this proposal, the target of PP2A would be cdc25 or wee1, or a regulator of cdc25 or wee1.

An alternative explanation is that PP2A regulates the state of threonine 161 phosphorylation, either directly or indirectly. Since threonine 161 phosphorylation is absolutely essential for the kinase activity of cdc2, the extent of phosphorylation on this site could be a rate-limiting step. In order

to better define the target(s) of PP2A, we needed to distinguish among these possibilities. In this study, we examined the effect of PP2A on the rate of phosphorylation and dephosphorylation of both the inhibitory and activating phosphorylation sites of cdc2. Since the level of PP2A activity appears to determine the length of the lag, the initial event, which opposes PP2A, and eventually overcomes PP2A, must occur during the lag. Therefore, we examined the effect of PP2A on these potential targets immediately after cyclin addition, up to the time of p34 ^{cdc2} activation. The results presented here indicate that PP2A affects neither cdc25 nor wee1/mik1 activities during the lag, but does affect threonine 161 phosphorylation. Furthermore, in a test of the hypothesis that a critical threshold level of active cdc2 kinase can activate a positive feedback loop turning on cdc25 and turning off wee1, we demonstrate the insufficiency of active cdc2 for this process.

RESULTS

INH is a conventional PP2A holoenzyme.

Our previous studies raised the possibility that INH might be a special form of PP2A. The finding that PP2A holoenzyme preparations from porcine cardiac tissue appeared to possess relatively little INH activity, whereas the catalytic subunit from the same tissue was active, led us to hypothesize that INH might be be a special embryonic form of PP2A that enabled the embryo to undergo rapid cleavage divisions. We took two approaches to this question: 1) Determining whether INH activity is unique to the oocyte and early embryo 2) Determining the molecular composition of INH. To address the first question, we prepared a crude extract from oocyte

and adult liver tissue, fractionated each by DEAE sepharose chromatography, and determined the peak of PP2A activity using a conventional substrate (myosin light chain). We then assayed the peak fractions for INH activity. If INH in the oocyte were a special form of PP2A, we would have expected oocyte PP2A fractions, when compared to adult liver PP2A fractions, to possess a higher level of INH activity relative to myosin light chain phosphatase activity. The comparison indicated that adult liver PP2A was just as active in the INH assay as the oocyte PP2A (data not shown). In addition, INH activity in both extracts copurified with the single major peak of PP2A activity on DEAE chromatography (data not shown). These results suggested that INH activity is not unique to the oocyte, and probably occurs throughout development; furthermore, INH appears to be the same as the major form of PP2A in oocytes as well as in the adult liver.

To address the second point, we purified INH from activated eggs by DEAE and tyrosine agarose chromatography, followed by sucrose density gradient centrifugation. Three major polypeptides copurified with INH, and corresponded in molecular weight to the known A (~64 kD) and B (~54 kD) regulatory subunit, and the catalytic subunit C (~37 kD) of conventional PP2A. The identity of the catalytic subunit was confirmed by immunoblotting with a monoclonal antibody against the catalytic subunit of PP2A (Lee *et al.*, 1991), and the identity of the polypeptide corresponding to the B subunit was confirmed by immunoblotting with a polyclonal sera directed against an N-terminal peptide of the Ba isoform of mammalian PP2A (Mayer *et al.*, 1991; data not shown). Gel purified material corresponding to the B subunit was obtained and cleaved with trypsin. The tryptic fragments were resolved by HPLC and subjected to amino acid sequence analysis (see materials and methods). Sequence information from

three tryptic fragments was obtained, and comparison to the cloned sequences of the B subunit isoforms of human PP2A (Mayer *et al.*, 1991) indicated a high degree of amino acid sequence identity between the Xenopus egg B subunit and the Bα isoform of human PP2A (figure 1). In conclusion, INH appears to be a conventional PP2A holoenzyme.

PP2A does not inhibit the tyrosine phosphatase during the lag.

Increasing the level of PP2A in the interphase extract by the addition of purified PP2A results in the inhibition of cdc2 activation (for instance, see figure 3, lower panel). p34 ^{cdc2} accumulates in the threonine 14 tyrosine 15 (T14Y15) phosphorylated form, suggesting that the inhibition by PP2A is due to a failure to activate the T14Y15 phosphatase and/or a failure to inactivate the T14Y15 kinase (Clarke *et al.*, 1993; our unpublished results). In order to assess whether this is due to direct regulation of the T14Y15 kinase(s) and phosphatase(s) by PP2A, we asked whether these enzyme activities are modulated by PP2A.

To measure the rate of tyrosine dephosphorylation on Y15 of cdc2, we generated a substrate that was radiolabelled stoichiometrically only on the inhibitory sites, Y15 and T14 (*materials and methods*). To generate this labelled substrate, we first activated cdc2 in an interphase extract with a non-degradable glutathione-S-transferase fusion of *X. laevis* cyclin B1 (GST-cyclin B), then isolated the active complex of p34 ^{cdc2} and cyclin on glutathione agarose beads. The active form of the kinase is phosphorylated on threonine 161 (T161) but not on the inhibitory T14Y15 sites as assessed by peptide mapping (Solomon *et al.*, 1990) and by probing here with an antiphosphotyrosine antibody (figure 2A, lane1). As we will demonstrate

below, the phosphate in T161 (unlabelled here) is resistant to the action of phosphatases in the extract, and therefore T161 cannot be labelled in subsequent incubations. To label the inhibitory sites, the active cdc2 kinase complex was mixed with an interphase extract in the presence of ^{32}P-γ-ATP but in the absence of additional free cyclin. This incubation resulted in the rapid inactivation of the glutathione agarose bound cdc2/cyclin complex and tyrosine phosphorylation of p34 ^{cdc2}, as determined by western blotting with the antiphosphotyrosine antibody (figure 2A, lane 2). Finally the phosphorylated substrate was reisolated on glutathione beads and eluted with free glutathione. The ^{32}P label in cdc2 (compare lanes 4 and 5 in figure 2B), as well as the immunoreactivity to the phosphotyrosine antibody (figure 2A, lane 3), was removed by incubating the complex with bacterially expressed and purified *Drosophila* cdc25. Since cdc25 is highly specific in its ability to dephosphorylate cdc2 on T14 as well as Y15, the removal of phosphate by cdc25 is indicative of phosphorylation on either or both T14 and Y15. Furthermore, since cdc25 is most likely responsible for both T14 and Y15 dephosphorylation, the rate of removal of the ^{32}P label from cdc2 should reflect the rate of removal of phosphotyrosine.

As expected from previous studies, the phosphorylated substrate is relatively stable in interphase extracts (k~.009 min^{-1}), and is more rapidly dephosphorylated in mitotic extracts (k~.052 min^{-1}, figure 2C). The addition of okadaic acid to an interphase extract in the absence of cyclin does not enhance the dephosphorylation of the substrate (k~.007 min^{-1}, figure 2C), suggesting that modulation of cdc25 activity requires the presence of cyclin in the extract. To measure the rate of tyrosine dephosphorylation during the normal course of mitotic activation, we added GST-cyclin B to an interphase extract that contained the labelled substrate. Thus the labelled cyclin/cdc2

complex was a tracer for the events occurring on the unlabelled cdc2. At various time points, an aliquot was taken to assay H1 kinase activity and another aliquot was removed to measure the phosphorylation state of the substrate after quenching with SDS sample buffer. As shown in figure 3, the rate of tyrosine dephosphorylation during the lag is low (k~.006 min^{-1}), reflecting the interphase rate (the difference in this rate compared to the rate given above for the interphase extract is due to variation in extracts, and does not reflect a difference in rates in the presence or absence of cyclin). At the time of cdc2 activation, the tyrosine phosphatase activity increases dramatically (to k~.061 min^{-1}), as has been demonstrated previously. When the same reaction was carried out in the presence of added PP2A (figure 3), the abrupt activation of the tyrosine phosphatase did not occur, and the H1 kinase activity of p34 ^{cdc2} remained the same as that in the interphase extract lacking added PP2A. This result confirmed our suspicion that PP2A ultimately inhibits the activation of cdc25. However, added PP2A had no effect on tyrosine phosphatase activity during the lag, which remained low (k~.006 min^{-1}), but no lower than in the control reaction (k~.006 min^{-1}). Thus during the lag phase, the tyrosine phosphatase activity is not further inhibited by PP2A, and therefore it appears that the elevation of tyrosine phosphatase activity during activation is a response to a different rate limiting triggering event.

PP2A does not activate the tyrosine kinase during the lag.

Since the tyrosine phosphatase was not affected by PP2A during the lag period, we next examined the effect of PP2A on the rate of tyrosine phosphorylation. It is sufficient to measure of the rate of accumulation of

phosphate if the rate of phosphorylation substantially exceeds that of dephosphorylation. During the lag, prior to the activation of the tyrosine phosphatase, the rate of tyrosine dephosphorylation was low enough (k~.006 min^{-1} compared to k~.07 min^{-1} for the rate of accumulation of phosphotyrosine) that the accumulation of phosphotyrosine in p34 ^{cdc2} could be used to assess the rate of tyrosine phosphorylation. At various times after cyclin addition, we measured the rate of tyrosine phosphorylation in p34 ^{cdc2} by immunoblotting the GST-cyclin B bound material with an anti-phosphotyrosine antibody (figure 4A; see *materials and methods*). We also probed the blot with a general cdc2 antibody (antiserum against the C-terminal peptide of *Xenopus* cdc2) to normalize the amount of phosphotyrosine in p34 ^{cdc2} to the amount of total p34 ^{cdc2} protein recovered (figure 4B). Figure 4a shows that in the control reaction, the rate of increase in tyrosine phosphate on cdc2 is linear for the first ten minutes (k~.07 min^{-1}). As can be seen by comparison of the reaction with and without added PP2A, the presence of elevated levels of PP2A does not affect the rate of tyrosine phosphorylation during the initial lag period following cyclin addition. Taken together, these results demonstrate that although PP2A eventually prevents the tyrosine dephosphorylation of p34 ^{cdc2}, PP2A does not modulate the tyrosine kinase or tyrosine phosphatase during the lag.

PP2A does not affect the rate of T161 dephosphorylation during cyclin activation of p34 ^{cdc2}

We had previously shown that the isolated and active p34 ^{cdc2}/cyclin complex can be directly inactivated by PP2A with the concommitant dephosphorylation of T161 (Lee *et al.*, 1991), suggesting that

PP2A could inhibit the activation of cdc2 by preventing the accumulation of T161 phosphate. A problem in the earlier experiments, however, was that a much higher concentration of PP2A was required to remove the T161 phosphorylation than was effective at blocking cdc2 activation. To address whether PP2A is the physiological phosphatase that dephosphorylates T161, we measured the rate of T161 dephosphorylation during cyclin activation of p34 ^{cdc2} in the extract and looked at the effects of minimal inhibitory levels of PP2A. To generate a p34 ^{cdc2} substrate that is phosphorylated solely on T161, we took advantage of the fact that purified CAK activates and phosphorylates reticulocyte lysate translated p34 ^{cdc2} on T161 (Solomon *et al.*, 1992; Fesquet et al., 1993; Poon, R.Y., 1993; Solomon *et al.*, 1993) in the presence of cyclin. GST-cyclin B was added to p34 ^{cdc2} (translated in a reticulocyte lysate) in the presence of highly purified CAK (*materials and methods*) and ^{32}P-γ-ATP. The phosphorylated complex was isolated on glutathione beads and eluted with free glutathione. In order to prove that the labelled 34 kd phosphoprotein represented T161 phosphorylated p34 ^{cdc2}, we demonstrated the absence of phosphate incorporation into the alanine 161 mutant (data not shown). To measure the rate of dephosphorylation, the T161 phosphorylated p34 ^{cdc2} substrate was added into interphase extracts at a level that, by itself, had no effect on the kinetics of activation of the endogenous p34 ^{cdc2} by cyclin B (data not shown). Upon addition of cyclin B, we examined the rate of loss of labelled phosphate from the substrate during the course of activation of the endogenous p34 ^{cdc2}. As shown in figure 5, the T161 phosphorylation is completely stable during the course of activation, even when activation is blocked by the addition of purified PP2A. Therefore, T161 dephosphorylation does not contribute to the regulation of p34 ^{cdc2} activation, and PP2A cannot directly dephosphorylate cyclin/p34 ^{cdc2} under these more physiological

conditions.

T161 phosphorylation is inhibited by PP2A.

Since the T161 residue is not dephosphorylated during the course of activation, either in the presence or absence of PP2A, we could measure the rate of T161 phosphorylation by quantitating the level of T161 phosphorylated p34 ^{cdc2} over time. To make this measurement, we took advantage of the fact that T161 phosphorylation confers a downward SDS gel mobility shift on p34 ^{cdc2}. By adding a trace amount of ^{35}S-methionine labelled p34 ^{cdc2} protein to the interphase extract, we could observe the rate of accumulation of the downward shifted form. A complicating feature of this analysis is that the downward mobility shift due to T161 phosphorylation is offset by an upward mobility shift conferred by T14Y15 phosphorylation. To avoid this complication, we used the mutant in the T14 and Y15 phosphorylation sites where those sites were converted to structurally similar but nonphosphorylatable residues, alanine 14 and phenylalanine 15 (A14F15). The low level of AF protein (~8% of the level of the endogenous cdc2) added to the reaction had no effect on the kinetics of cdc2 activation (data not shown), and therefore acted simply as a marker for the state of T161 phosphorylation. At each time point, in addition to taking samples for analysis of T161 phosphorylation, we also measured the H1 kinase activity in the extract. As shown in figure 6A, the rate of T161 phosphorylation is significantly slowed by the addition of 130 nM PP2A (k~.15 min^{-1} to k~.11 min^{-1}). At higher levels of PP2A, the reaction is delayed even further (figure 6B), suggesting that the level of PP2A in the extract determines the level of T161 phosphorylating activity. Consistent with this hypothesis, blocking

PP2A activity in the extract with 1 μM okadaic acid accelerates the T161 phosphorylation reaction (figure 6C).

Since the antagonistic relationship between cyclin and PP2A is central to the mechanism of the trigger, a rate limiting event that is part of the trigger should be stimulated by cyclin in addition to being inhibited by PP2A. As shown in figure 6D, raising the level of cyclin added to the reaction offsets the inhibitory effect of added PP2A on the initial rate of T161 phsophorylation. We can conclude from this result that cyclin and PP2A oppose one another in the regulation of T161 phosphorylation of cdc2.

Previous studies showed that T161 phosphorylation requires prior association of p34 ^{cdc2} with cyclin. In a separate experiment, we asked whether PP2A had an effect on the association of p34 ^{cdc2} with cyclin, which could potentially account for the delay in T161 phosphorylation. To measure the rate of association, control interphase extracts or interphase extracts to which PP2A was added, were spiked with ^{35}S-A14F15 p34 ^{cdc2}. GST-cyclin B prebound to glutathione agarose beads was then added. The complex could be readily isolated and the cyclin bound p34 ^{cdc2} quantitated. As indicated in figure 7, the rate of association (k~.07 min^{-1}) was not significantly affected by the presence of added PP2A (k~.06 min^{-1}). However, the rate of T161 phosphorylation in the same reaction was inhibited by 50% (k~.08 min^{-1} to k~.04 min^{-1} in the presence of added PP2A). Therefore, PP2A does not delay the rate of association of p34 ^{cdc2} and cyclin B, but does inhibit the rate of T161 phosphorylation.

T161 phosphorylation stabilizes the p34 ^{cdc2}/cyclin B complex.

In the process of studying the affect of PP2A on p34 ^{cdc2}/cyclin

complex formation, we found that although the rate of association between p34 ^{cdc2} and cyclin B is not affected by T161 phosphorylation, T161 phosphorylation lowers the rate of dissociation of the complex. As shown in figure 8, a comparison of the stability of the complex formed between cyclin B and p34 ^{cdc2} phosphorylated on T161 with the complex formed between cyclin B and p34 ^{cdc2} that cannot be phosphorylated on T161 (provided by the alanine 161 p34 ^{cdc2} protein), showed that the phosphorylated p34 ^{cdc2} was completely stable while the complex containing the unphosphorylatable p34 ^{cdc2} dissociated at a significant rate with successive 20-fold dilutions. Therefore, the phosphorylation of p34 ^{cdc2} on T161, or some other consequence of T161 phosphorylation, stabilizes the interaction between p34 ^{cdc2} and cyclin B. This result may explain the discrepancies among different investigators with respect to the ability of the unphosphorylated form of p34 ^{cdc2} to bind cyclin. The result would depend on the stringency of the binding assay, although the significance *in vivo* may be minimal since the concentrations of p34 cdc2 and cyclin in the cell are apt to be higher than the estimated dissociation constant of ~1nM.

AF p34 ^{cdc2} is sensitive to inhibition by PP2A.

The results presented so far demonstrate that PP2A affects only one known reaction during the lag, that of T161 phosphorylation. This would indicate that PP2A exerts its inhibitory effect on the activation of p34 ^{cdc2} by modulating the T161 phosphorylation reaction. If, in fact, PP2A were acting solely through the regulation of T161 phosphorylation, we would predict that the A14F15 p34 ^{cdc2} protein, which cannot be inhibited by tyrosine phosphorylation, to retain sensitivity to inhibition by PP2A. To test this

hypothesis, wild type and A14F15 p34 ^{cdc2} containing the HA epitope tag at the C-terminus were translated in reticulocyte lysates and added to the interphase extract such that the exogenous AF p34 ^{cdc2} comprised about 50% of the total p34 ^{cdc2}. Thirty minutes following cyclin B addition, the exogenous p34 ^{cdc2} was specifically removed by immunoprecipitation with a monoclonal antibody against the HA epitope and its H1 kinase activity assessed. Figure 9A compares the H1 kinase activity achieved by the wild type protein by 30 minutes in the presence of added PP2A (compare lanes 3 and 4 to lane 2) to that achieved by the A14F15 protein in the presence of the same levels of PP2A (compare lanes 6 and 7 to lane 5). At these concentrations of PP2A, the A14F15 p34 ^{cdc2} is, in fact, sensitive to PP2A. This result is consistent with our finding that PP2A can regulate p34 ^{cdc2} through inhibiting the phosphorylation of T161 and seems to leave no role for cdc25 in the activation process. Yet the A14F15 mutant shows precocious activation (data not shown), suggesting that the inhibitory sites can be rate limiting. We therefore carefully examined the sensitivity of the A14F15 and wild type protein to PP2A. Figure 9B shows a dose response of both forms of p34 ^{cdc2} to a range of PP2A concentrations. The results indicate that although the activation of the A14F15 protein shows some sensitivity to PP2A, it is much less sensitive than the wild type protein. Whereas the activation of the normal p34 ^{cdc2} is completely blocked by the addition of 100 nM PP2A, the A14F15 protein is able to partially activate even in the presence of 400 nM PP2A. We conclude, from these results, that although the rate of T161 phosphorylation might normally be an important determinant of the kinetics of cdc2 activation, it is not the only determinant and that PP2A also regulates p34 ^{cdc2} through the inhibitory phosphorylation sites.

There is an apparent paradox. PP2A seems to have no effect on

the rate of tyrosine phosphorylation or dephosphorylation during the lag, yet the kinetics of activation seem to be strongly influenced by the availability of the inhibitory phosphorylation sites (or by the addition of excess cdc25, see Gautier *et al.*, 1991). One way of reconciling these results is that PP2A may regulate the tyrosine kinase and phosphatase activities indirectly, through the regulation of T161 phosphorylation. By delaying T161 phosphorylation during the lag, PP2A might delay indirectly the activation of the tyrosine phosphatase and the inactivation of the tyrosine kinase. However, in the absence of the inhibitory phosphorylation sites (provided by the A14F15 protein), the rate of T161 phosphorylation is the only limiting reaction. In this situation, although PP2A inhibits this reaction, the consequences are not as dramatic in the absence of the inhibitory phosphorylations.

Active cdc2 is not sufficient to turn on its own activation.

These results suggest that the activation process may involve several intermediates. For example, Solomon *et al.* initially proposed that the accumulation of a critical threshold level of active p34 ^{cdc2}, phosphorylated on T161 but not on T14Y15, might be a prequisite to activating a positive feedback loop which would subsequently activate the bulk of the p34 ^{cdc2} by tyrosine dephosphorylation. The data presented here might suggest that PP2A, by slowing the rate of T161 phosphorylation, would delay the accumulation of the critical threshold level of active p34 ^{cdc2} molecules, and thus prevent the subsequent activation of the bulk of the p34 ^{cdc2}. According to this proposal, the rate of T161 phosphorylation and consequently the rate of accumulation of active p34 ^{cdc2} during the lag would comprise the rate-determining step or switch to activated cdc2. A simple test of such a model

would be to add a superthreshold level of active p34 ^{cdc2}/cyclin B complex to an interphase extract. The added kinase should bypass the initial requirement and result in the activation of the positive feedback loop, resulting in a stable mitotic state. (The use of a nondegradable cyclin, provided by the glutathione S-transferase portion of GST-cyclin B, could prevent the subsequent cyclin degradation). We therefore added a superthreshold level of active p34 ^{cdc2}/cyclin B complex to an interphase extract (figure 10A, lane 1). Surprisingly, most of the H1 kinase activity of the added complex was quickly lost (lane 2). Inactivation occurred even when the level of the added complex exceeded the amount that would have been generated in the recipient extract by the addition of an equivalent amount of free cyclin (see below). The activated p34 ^{cdc2}/cyclin complex was thus insufficient to turn on the enzymes required to maintain cdc2 in the active state. The inactivation of the H1 kinase could be subsequently reversed by the addition of bacterially expressed and purified cdc25 to the inactivated and reisolated complex (figure 10A, lane 4), demonstrating that the inactivation was reversible, not as if it were caused by proteolysis, but rather through phosphorylation on T14Y15.

We compared the effect of cyclin bound to active cdc2 with that of free cyclin to ensure that the level of active complex we were adding exceeded the threshold levels of cyclin that would normally be sufficient to activate stable levels of cdc2 activity. GST-cyclin B, pre-bound to glutathione beads, was incubated in interphase extracts for different lengths of time. At the indicated times, we isolated the cyclin on the glutathione beads and either assayed for the H1 kinase activity on the beads (figure 10B, lanes 1-4), or incubated the beads in a second interphase extract for 20 or 40 minutes (lanes 5-10). We then reisolated the complex and assayed for H1 kinase activity. Lanes 1-4 indicate the time course of H1 kinase activation in the first

incubation, and lanes 5-10 indicate the H1 kinase activity remaining on the beads at each time point after incubation in a second interphase extract, in the absence of any additional cyclin. As shown in lanes 1-4, H1 kinase activity begins to increase during the first incubation at 20 minutes, reaches a maximum level by 40 minutes, and remains stable to 80 minutes. As shown in lanes 5 and 6, at a time when cyclin has no associated H1 kinase activity in the first extract (lane 1), it is fully capable of activating a second extract. However, by 20 minutes in the first extract (lane 2), it is no longer capable of activating a second interphase extract (lanes 7 and 8). Instead, most of the H1 kinase activity associated with cyclin during the incubation in the first extract (lane 3) is rapidly lost upon addition to the second extract (lanes 9 and 10). These findings demonstrate that, although the presence of a critical threshold level of active cdc2 may be rate-limiting, it is not sufficient to activate the enzymes that maintain its own activity. In fact, as cyclin binds to p34 ^{cdc2} and turns on the kinase activity of cdc2, it appears to lose the capacity to induce a stable mitotic state. As shown in figure 10A, lane 3, the H1 kinase activity of the p34 ^{cdc2}/cyclin complex is not lost when added to a second interphase extract containing 1μM okadaic acid. Thus PP2A appears to act at a step distinct from the initial accumulation of the active cdc2/cyclin complex.

The addition of a non-degradable cyclin to an interphase extract leads to the activation and stable maintenance of cdc2/cyclin in an active state. Since the ability to drive an interphase extract into M phase does not reside in the fully actived cdc2/cyclin complex, we asked whether a mitotic extract that has been depleted of the active cdc2/cyclin complex retains the ability to stabilize the active complex. For this, we used glutathione agarose beads to deplete a stable mitotic extract of the cdc2/cyclin complex (figure 10C, lanes 3-6). In order to probe the state of the depleted extract, we added back

the active complex (lane 1) after incubating the depleted extract for various times. As shown in figure 10C, the ability to stabilize the active cdc2/cyclin complex is lost rapidly (within 2 min, lanes 3-6) from the mitotic extract upon depletion of the active complex, suggesting that although the cdc2/cyclin complex is not sufficient by itself to initiate all of the events required for its stabilization, it is necessary for its own stabilization. Furthermore, since the activity required for the stabilization of the cdc2/cyclin active complex is lost upon separation of the active complex from a mitotic extract, we conclude that the continued interaction of the active complex with some unidentified factor(s) is required for the maintenance, and probably also the generation, of the mitotic state.

DISCUSSION

The accumulation of a critical threshold level of cyclin protein triggers the interphase to mitotic transition during the first *Xenopus* embryonic cell cycle. The cyclin threshold is directly determined by the level of PP2A; therefore, PP2A must limit the action of cyclin such that at subthreshold concentrations, the extract remains in interphase, whereas at above threshold concentrations, the extract rapidly enters mitosis. This antagonistic relationship between cyclin and PP2A is essential for the switch-like response to a continously increasing pool of cyclin, *i.e.*, in the absence of PP2A activity, cyclin activates cdc2 proportionately and without a significant lag. Central to understanding the all or none mechanism for entering mitosis is the identification of the link between cyclin and PP2A.

We have used purified PP2A from *Xenopus* eggs and *Xenopus* interphase extracts to explore the mechanism by which PP2A and cyclin

oppose one another. The mitotic state is produced by the activation of the cdc2/cyclin protein kinase complex. Since cyclin is an essential component of this key regulator, the link between cyclin and PP2A should lie somewhere along the pathway to cdc2 activation. Prior to this study, we knew in a broad sense the sequence of events following cyclin addition. Cyclin associated with cdc2 to form a complex that was inactive because it lacked an essential phosphorylation on the activating site, T161. Cyclin binding allowed phosphorylation on T161, but it also simultaneously induced phosphorylation on two inhibitory residues Y15 and T14. Below the threshold concentration of cyclin, the complex remained in the triply phosphorylated form and was inhibited. Above this threshold, cyclin catalyzed a series of events, which after a lag, led to a switch in the balance between the tyrosine kinase and phosphatase activities such that the complex was abruptly dephosphorylated and activated. Thus the activities that dephosphorylated the tyrosine 15 residue and that phosphorylated the tyrosine 15 residue were markedly different before and after the mitotic transition. There was no obvious modulation of the activating phosphorylation on T161 throughout the transition. In order to identify, among these phosphorylation and dephosphorylation reactions on cdc2, the triggering event, we compared each reaction pathway under two conditions, one in which cyclin exceeded the threshold, and one in which cyclin was below threshold. These conditions could be achieved by varying slightly (2-3 fold) the ratio of cyclin to PP2A.

In past studies, the problem of identifying the trigger, *ie* that event that was sensitively regulated by cyclin and PP2A, was complicated by two major issues. The first complication arises in distinguishing those events that are a direct target of PP2A and are potentially rate limiting for the

initiation of the transition, from those events that occur after the transition, downstream of the trigger. By blocking the initiation of the transition, PP2A ultimately affects all of the events that would accompany the transition. The second complication arises from the difficulty in assessing the physiological relevance of partial reactions that are catalyzed among purified components, particularly for an enzyme (like PP2A) that exhibits a broad substrate specificity *in vitro,* and little selectivity at high concentrations. The present study is an attempt to deal with both of these issues.

The problem of cause and effect is illustrated by the relationship between PP2A and cdc25. The addition of okadaic acid to an interphase extract and cyclin results in the premature conversion of cdc25 to the highly phosphorylated and active, mitotic form. Similarly, the addition of purified PP2A to an interphase extract with cyclin blocks the activation of cdc25. These results imply that PP2A negatively regulates the activity of cdc25. But whether cdc25 could be part of the trigger for the transition depends on whether PP2A directly inhibits cdc25, or whether PP2A inhibits cdc25 by preventing the transition from occurring. In order to separate the primary effects of PP2A from those effects that occur as a consequence of blocking the initiating event, we examined the effect of PP2A on cdc25 activity during the lag, between the time of cyclin addition and the time of the transition. The lag period is the relevant period for a trigger since this is the time when either the sufficiency or insufficiency of the cyclin level is being evaluated by the kinetics of the reaction pathways leading to the activation of cdc2. Our results suggest that although PP2A may inhibit cdc25 after the transition, the level of PP2A does not modulate cdc25 activity prior to the transition. It appears, therefore, that the inhibitory effect of PP2A on cdc25 is an indirect consequence of the inhibitory effect of PP2A on the trigger for

mitosis. During the transition itself, changes in the activity of cdc25 may be important in adding an autocatalytic acceleration to the acquisition of the mitotic state, as suggested by Solomon *et al.*, (1990).

Similarly, we can rule out a role for changes in tyrosine phosphorylation in the trigger for mitosis by studying the effect of PP2A on the tyrosine kinase activity during the lag. Previous studies showed that the addition of okadaic acid to an extract arrested in interphase, with high tyrosine kinase activity, led to a 10 fold reduction in the tyrosine kinase activity (Smythe and Newport, 1992), suggesting that PP2A regulated the tyrosine kinase. We find no significant difference in the tyrosine kinase activity, during the lag, in the presence of elevated PP2A activity. Therefore the effect of PP2A on the tyrosine kinase must also be downstream of some other regulator after the transition. Since we did not look directly at T14 phosphorylation, it remains a possibility that the T14 kinase is modulated by PP2A, independently of Y15 phosphorylation. However, Norbury *et al.* (1991) have shown that abolishing either one of the inhibitory T14 and Y15 residues for phosphorylation had no discernible effect on the kinetics of cdc2 activation, suggesting that either T14 or Y15 phosphorylation is sufficient for conferring negative regulation on cdc2. In agreement with their report, we find that both A14Y15 and T14F15 cdc2 molecules are as sensitive to inhibition by PP2A as is the wild type protein (unpublished results). In light of this observation, it is unlikely that down regulation of the T14 kinase, even if modulated independently of the Y15 kinase, would comprise a rate limiting step, since dephosphorylation of T14 in the absence of Y15 dephosphorylation would not activate cdc2. In summary, it appears that a change in the balance of the kinase(s) and phosphatase(s) that control the inhibitory sites on cdc2 is not part of the trigger, but rather a consequence of

the trigger.

The second complication described above, that of determining which reactions are physiologically relevant, became clear when we initially examined the effect of PP2A on T161 dephosphorylation. We had previously demonstrated the ability of purified PP2A to directly dephosphorylate T161 on cdc2 and to inactivate it. Based on this result, we suggested that perhaps PP2A regulated cdc2 by dephosphorylating cdc2 on T161. We noted, however, that micromolar concentrations of PP2A were required to see this effect, whereas nanomolar concentrations of PP2A were sufficient to block the initial activation of cdc2.

The importance of the T161 dephosphorylation reaction is best evaluated in a complex extract, under more physiological conditions. In these conditions T161 dephosphorylation does not occur at a measureable rate. This is not to suggest that T161 dephosphorylation is not important at some step of mitosis. Lorca *et al.* (1992) have demonstrated a requirement for T161 dephosphorylation on cdc2, subsequent to cyclin degradation, for the inactivation of cdc2. Thus dephosphorylation of T161 could potentially play a regulatory role in the exit from M phase, although a situation in which it is rate limiting has yet to be observed.

Despite its broad substrate specificity *in vitro*, when PP2A is added to an interphase extract at levels that just prevent the cyclin triggered transition, it has no discernible effect on most of the reactions governing cdc2 activity. Neither cyclin binding to cdc2, nor the phosphorylation or dephosphorylation reactions on Y15 are affected by PP2A. But at the same time, the T161 phosphorylation reaction is sensitively modulated by PP2A. Therefore, under more physiological conditions, in limiting amounts, PP2A exhibits a degree of substrate specificity. The addition of PP2A to the extract

lowers the initial rate of T161 phosphorylation in a dose-dependent manner. Furthermore, this inhibitory effect can be counteracted by increasing the level of cyclin added to the reaction, arguing in favor of the T161 phosphorylation reaction being part of the link between cyclin and PP2A. Whether the regulation of T161 phosphorylation occurs at the level of the kinase that phosphorylates T161 is not known. Solomon *et al.* (1992) have shown in fact that the activity of the T161 kinase (CAK of which MO15 has been identified as the catalytic subunit) is not changed between interphase and mitosis. The regulation by PP2A may set a baseline value for CAK activity that is independent of cell cycle states. As expected, inhibition of PP2A activity in the extract with okadaic acid accelerates T161 phosphorylation. Formally this could be an indirect consequence of the acceleration of the transition; however, the rate of T161 phosphorylation appears to be linear and shows no discontinuity through the transition, suggesting that the reaction is not stimulated during the transition. Thus the acceleration by okadaic acid probably reflects a more direct effect of PP2A on the T161 phosphorylation reaction.

Based on these results, we might expect that cdc2 mutants insensitive to the inactivating phosphorylations (the A14F15 mutant) should show some sensitivity to regulation of the T161 phosphorylation rate. In fact, we find the activation of the A14F15 protein to be sensitive (although not as strongly as the wild type protein) to the level of PP2A in the extract. However, the decreased sensitivity of the A14F15 protein suggests that somehow PP2A regulates the level of tyrosine phosphorylation, even though it seems to have no direct effect on those reactions. We are left with the conclusion that PP2A exerts an inhibitory effect on the pathway controlling Y15 phosphorylation at least in part through its effect on T161

phosphorylation, which suggests to us that T161 phosphorylation might be upstream to the switch in tyrosine kinase and phosphatase activities which in turn participate in the transition. Thus T161 phosphorylation, which appears to be modulated by cyclin and PP2A, is potentially a rate limiting step in the initiation of mitosis. However, definitive proof of the importance of T161 phosphorylation in limiting the trigger clearly awaits the ability to specifically modulate the rate of this reaction independently of PP2A.

The kinetic data on cdc2 activation fits nicely with a model in which PP2A sets the threshold through its regulation of T161 phosphorylation. Such a model was initially proposed by Solomon *et al*, and we call it the positive feedback loop model. This model postulates that cyclin addition leads to the proportionate accumulation of a low but critical threshold level of active cdc2/cyclin complexes. This active fraction of cdc2 then triggers a switch in the balance between tyrosine kinase and tyrosine phosphatase activities, which subsequently leads to the activation of the bulk of the cdc2/cyclin complexes initially held inactive by tyrosine phosphorylation. By delaying the onset of T161 phosphorylation, PP2A could delay the accumulation of a critical threshold level of active cdc2. As a consequence, tyrosine phosphorylation would outcompete T161 phosphorylation, not allowing the active complexes (the trigger) to form. This model places T161 phosphorylation central to the trigger, as the rate limiting step for M phase initiation.

A prediction of the positive feedback loop model is that the addition of the activated cdc2/cyclin complex to an interphase extract would bypass the requirement for the initial trigger and directly induce the mitotic state. To our surprise, the simple test of the model failed. We found that adding the fully activated cdc2/cyclin complex to an interphase extract, rather

than accelerating the conversion of the interphase extract into a mitotic extract, resulted in the inactivation of the complex, suggesting that the active cdc2/cyclin complex failed to activate cdc25 and/or inactivate the tyrosine kinase, a prerequisite for a stable mitotic state. In agreement with these results, Gonzalez-Kuyvenhoven *et al.* (manuscript submitted) have demonstrated the inability of the cdc2/cyclin complex to mitotically phosphorylate cdc25 in an interphase extract. Whether or not the tyrosine kinase is similarly maintained in the interphase state remains to be determined, but the rapid kinetics of inactivation would suggest that the tyrosine kinase is active. The failure of the active cdc2/cyclin complex to drive an interphase extract into a stable mitotic state (unless PP2A is first inactivated) raises several important points. First, a positive feedback loop in which cdc2 turns on its own activators cannot by itself explain mitotic initiation. Second, although T161 phosphorylation might dictate when entry into M phase occurs under certain conditions, it cannot be the only rate limiting step. Third, there must be an additional target of PP2A.

While cyclin added to the interphase extract can initiate all of the events required for mitotic entry, cyclin bound to cdc2 and fully activated as a protein kinase complex cannot. Since the only known end product of cyclin addition is its fully activated complex with cdc2, the finding that the cdc2 complex is unstable is unexpected, and appears to undermine the importance of the active cdc2/cyclin complex in triggering mitotic initiation. However, we show that removal of active cdc2 from a mitotic extract causes the rapid conversion of the mitotic extract to an interphase extract, strongly suggesting that the cdc2/cyclin complex plays an active role in the maintenance of the mitotic state (as shown by Dunphy *et al.*, 1988). Since the enzymes stabilizing the mitotic forms of cdc25 and tyrosine kinase are

inactivated upon separation of the active cdc2 complex from the mitotic extract, and yet cdc2 itself does not retain the ability to convert the interphase forms to their mitotic counterparts, the active cdc2/cyclin complex must act in concert with other unidentified factors to initiate the conversion of cdc25 and the tyrosine kinase to the mitotic state. Thus cyclin addition may trigger the initiation of mitosis by activating a multi-component system (as has been suggested for MPF itself, Kuang *et al.*, 1991). We do not know what form of cyclin is responsible for activating the partner to the cdc2/cyclin complex in the trigger. A candidate might be a complex between the unphosphorylated form of cyclin and cdc2 that is present in the early phases of activation, to be replaced by the phosphorylated form of cyclin in the mitotic state. However, it could also be free cyclin, or cyclin bound to a kinase other than cdc2.

In summary, we began this study with the intent to identify the link between cyclin and PP2A in hopes of understanding the mechanism of the trigger for mitosis. Our results suggest that T161 phosphorylation and the accumulation of active cdc2 during the lag may be part of the trigger for mitotic initiation, and that the switch in tyrosine kinase and tyrosine phosphatase activities reside downstream of the initiating event. However, our results also suggest that the accumulation of active cdc2/cyclin complexes during the lag is only one of the events required for triggering entry into mitosis. The identification of the other reaction pathway(s) initiated by cyclin, and opposed by PP2A, will lead to a more complete understanding of the trigger for mitosis.

FIGURE LEGENDS

Figure 1. Sequence comparision of the B subunit of INH with the Bα subunit of human PP2A. The sequences of three tryptic fragments obtained from the B subunit of INH are aligned with the amino acid sequence of the Bα isoform of human PP2A (Mayer *et al.*, 1991).

Figure 2. Generation of the tyrosine phosphorylated cdc2 substrate (as described in *materials and methods*). (A) Phosphotyrosine blot of (lane 1) glutathione agarose-bound cyclin/cdc2 complex in the first stage, (lane 2) the complex after the second stage incubation, and (lane 3) the complex following a 30 minute incubation at room temperature with 3 μM bacterially expressed*Drosophila* cdc25 in XB. (B) The complex is ^{32}P labelled on tyrosine 15 and probably threonine 14. The ^{32}P label in the complex following the second stage incubation (lane 4) is efficiently removed upon a 30 minute incubation with 3 μM *Drosophila* cdc25 in XB (lane 5). (C) The substrate is dephosphorylated rapidly in mitosis but not in interphase or in interphase in the presence of 1μM okadaic acid. 6 μl of the ^{32}P labelled substrate was mixed with 9 μl of a mitotic extract (7.5 μl interphase extract + 1.5 μl of 300 nM GST-cyclin B and preincubated for 30 min at room temperature), interphase extract (7.5 μl interphase extract + 1.5 μl XB), or interphase extract in the presence of 1μM okadaic acid (7.5 μl interphase + 1.5 μl of 20 μM okadaic acid). At the indicated times, 2 μl of the mixture was removed and quenched in SDS sample buffer. The samples were resolved by SDS-PAGE and visualized by a phosphorimager system (Molecular Dynamics) for quantitation.

Figure 3. PP2A does not affect the tyrosine phosphatase activity during the lag. 4 µl of the tyrosine phosphorylated substrate was mixed with 12 µl of interphase extract + 4 µl of 0.5 µM GST-cyclin B in the presence of 20 µl of either 400 nM purified PP2A in XB or XB alone. After the indicated times at room temperature, 3 µl of the mixture was removed and quenched in SDS sample buffer while 3 µl was bound to 10 µl p13 ^{suc1} beads on ice (Solomon *et al.*, 1990) and assayed for H1 kinase activity (*materials and methods*). The graph depicts the percent loss of ^{32}P label from the substrate with time, while the lower panel shows the H1 kinase activity at each corresponding time.

Figure 4. PP2A does not affect the tyrosine kinase activity during the lag. 24 µl of interphase extract was mixed with 16 µl of XB containing 125 nM GST-cyclin B in the presence of 40 µl of either 200 nM purified PP2A in XB or XB alone. At the indicated times, 20 µl of the mixture was removed and bound to 18 µl of glutathione agarose beads on ice for 15 min. The beads were briefly rinsed with 0.5 ml XB and mixed with SDS sample buffer. To ensure that activation occured in the control reaction but not in the PP2A treated reaction, the H1 kinase activity was assessed after 30 min in each reaction (data not shown). Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with (A) the 4G10 anti-phosphotyrosine antibody and then reprobed with (B) an antiserum directed against the C-terminal peptide of *Xenopus* cdc2.

Figure 5. Threonine 161 dephosphorylation does not occur at a measureable rate, in the presence or absence of added PP2A. 16 µl of the ^{32}P labelled threonine 161 phosphorylated cdc2 substrate (see*materials and methods*) was mixed with 12 µl of interphase extract + 4 µl of 0.5 mM GST-cyclin B in the

presence of 8 µl of either 500 nM purified PP2A in XB or XB alone. At the indicated times, 8 µl of the reaction mixture was removed for immunoprecipitation with the anti-HA antibody on ice (*materials and methods*), and 1 µl was removed and diluted on ice for H1 kinase assay. The immunoprecipitated cdc2 was visualized by SDS-PAGE and autoradiography, as well as a phosphorimager system for quantitation. The top panel depicts the ^{32}P labelled substrate with time and the lower panel shows the H1 kinase activity at each indicated time.

Figure 6. PP2A inhibits threonine 161 phosphorylation. (A) 0.4 µl of reticulocyte lysate, that had been programmed with ^{35}S-methionine and the A14F15 cdc2 mRNA (Gautier *et al.*, 1992) and containing 10 ng/µl cdc2 protein, was pre-incubated with 5 µl of interphase extract. After 10 min at room temperature, 1 µl of 0.5 mM GST-cyclin B was added in the presence of 4 µl of either 320 nM purified PP2A in XB or XB alone. At the indicated times, 0.5 µl of the reaction mixture was quenched with SDS sample buffer, and 1 µl of the reaction was diluted on ice for H1 kinase assay. The ^{35}S labelled protein mixture was separated by SDS-PAGE (10%) to resolve the threonine 161 phosphorylated cdc2 from the unphosphorylated cdc2, and visualized by enhanced autoradiography as well as a phosphorimager system for quantitation. The upper panel depicts the accumulation of the shifted, phosphorylated ^{35}S labelled cdc2, and the lower panel shows the H1 kinase activity in the reaction at the indicated times. (B) At a fixed concentration of cyclin, the initial rate of threonine 161 phosphorylation is determined by the level of PP2A in the extract. A graphical representation of an experiment such as described in (A) at three different concentrations of PP2A. The endogenous level of PP2A in the extract is estimated to be ~160 nM (data not

shown). The initial velocity (arbitrary units here) of T161 phosphorylation refers to the relative rate of the reaction during the initial 10 min. (C) Okadaic acid accelerates threonine 161 phosphorylation when it induces the premature activation of cdc2. 0.5 µl of 10 ng/µl cdc2-containing reticulocyte lysate was pre-incubated in 6 µl of an interphase extract. After 10 min at room temperature, 2 µl of 0.4 mM GST-cyclin B + 12 µl of XB or XB containing 1.7 µM okadaic acid was added. At the indicated times, 2 µl of the reaction mixture was quenched with SDS sample buffer and 1 µl of the mix was diluted on ice for H1 kinase assay. The radioactivity in H1 was measured by directly counting the gel bands in a scintillation counter. (D) Increasing the cyclin level counteracts the inhibitory effect of PP2A on the initial rate of T161 phosphorylation. 2.5 µl of interphase extract that had been pre-incubated with cdc2-containing reticulocyte lysate as above was incubated with 2.5 µl of 800 nM PP2A in XB or 2.5 µl of XB in the presence of 52, 140 or 350 nM GST-cyclin B (final concentration). At the indicated times, 0.5 µl of the reaction mixture was removed and quenched with SDS-sample buffer. The percent of T161 phosphorylated form of cdc2 was resolved and quantitated as described in (A).

Figure 7. While it inhibits threonine 161 phosphorylation, PP2A does not inhibit the association of cdc2 and cyclin. 100 ng of GST-cyclin B was pre-bound to each 8 µl aliquot of glutathione agarose beads (60 min on ice). 6 µl of interphase extract that had been pre-incubated for 10 min at room temperature with 0.5 µl of reticulocyte lysate containing 10 ng/µl ^{35}S-labelled A14F15 cdc2 protein, was added to each aliquot of beads in the presence of 10 µl of either 500 nM purified PP2A in XB or XB alone. At the indicated times, the beads were rinsed once with 200 µl XB and mixed with SDS sample buffer.

Of the total cyclin-bound cdc2, the threonine 161 phosphorylated and unphosphorylated forms of cdc2 were resolved by SDS-PAGE (10%) and quantitated on a phosphorimager system.

Figure 8. Threonine 161 phosphorylation stabilizes the cdc2/cyclin complex. 100 ng of GST-cyclin B was pre-bound to 8 µl aliquots of glutathione agarose beads. 6 µl of interphase extract, that had been pre-incubated with 0.5 µl of reticulocyte lysate containing 10 ng/µl of ^{35}S-labelled wild type or A161 (that cannot be phsophorylated on threonine 161) cdc2 protein, and 10 µl of XB was added to each aliquot of the cyclin-bound beads. After 30 min at room temperature to allow mitotic entry and thus threonine 161 phosphorylation of the wild type cdc2, each aliquot of beads was washed 1, 2, 3 or 4 times with 200 µl of XB. The proteins remaining on the beads were then mixed with SDS sample buffer and resolved on SDS-PAGE. The cyclin-bound cdc2 was visualized and quantitated using a phosphorimager system.

Figure 9. (A) Activation of the A14F15 cdc2 protein is inhibited by PP2A. HA epitope-tagged wild type (lanes 2-4), A14F15 (lanes 5-7) and A161 (lane 1 as a negative control) cdc2 were translated in reticulocyte lysates to ~10 ng/µl. 2 ml of reticulocyte lysate was mixed with 3 µl of interphase extract and 1 µl of 0.5 µM GST-cyclin B in the presence of 5 µl of 800 nM purified PP2A in XB (lanes 3 and 6), 400 nM PP2A (lanes 4 and 7), or XB alone (lanes 1, 2 and 5). After a 30 min incubation at room temperature, each sample was mixed with 10 µl of anti-HA antibody-bound protein A sepharose beads, and incubated on ice for 30 min. The beads were then washed with 3 x 1 ml (XB + 0.5 M NaCl + 1% NP40 + 1µM okadaic acid) x 1hr at 4° C with rotation, followed by 2 x 1 ml brief washes in XB. The immunoprecipitates were then assayed for H1 kinase

activity. (B) Activation of the A14F15 cdc2 protein is less sensitive to PP2A. 2 µl of reticulocyte lysate containing the wild type or A14F15 cdc2 protein were mixed with 3 µl of interphase extract and 1 µl of 0.5 µM GST-cyclin B in the presence of 5 µl of 0, 100, 200, 400 or 800 nM purified PP2A in XB. After 30 min, the HA epitope-tagged cdc2 was immunoprecipitated from each reaction as in (A) and assayed for H1 kinase activity. 100% refers to the amount of activity achieved by 30 min in the absence of added PP2A.

Figure 10. The active cdc2/cyclin complex cannot convert an interphase extract to the mitotic state in the absence of excess free cyclin. (A) The active complex becomes inactivated in an interphase extract. 20 µl of interphase extract was mixed with 20 µl of 100 nM GST-cyclin B in XB at room temperature for 30 min to allow activation of cdc2. After 30 min, 10 µl aliquots were removed and each bound to 10 µl of glutathione agarose beads for 15 min at room temperature. Each aliquot of beads was rinsed with 0.5 ml of XB and incubated with 5 µl of interphase extract +5 µl of XB (lanes 2 and 4), with 5 µl of interphase extract + 5 µl of XB containing 2 µM okadaic acid (lane 3), or with 10 µl of XB alone (lane 1). After 15 min at room temperature, each sample was washed once in XB^{++} and once in XB. The sample in lane 4 was further treated with 10 µl of 3 µM bacterially expressed *Drosophila* cdc25 for 30 min at room temperature and then washed as described. Finally, each aliquot was assayed for H1 kinase activity. (B) The inability of the active cdc2/cyclin complex to drive the interphase to mitotic transition is not a threshold effect. 150 ng of GST-cyclin B were bound to each of 10-10 µl aliquots of glutathione agarose beads on ice for 60 min. The beads were briefly rinsed in 200 µl XB, then incubated with 10 µl of a 1:1 diluted interphase extract in XB for 0 (lanes 1, 5 and 6), 20 (lanes 2, 7 and 8), 40 (lanes 3, 9 and 10) or 80 (lane 4) min at

room temperature. At the respective times, the beads were rinsed with 200 ml of XB and left untreated (lanes 1-4) or incubated with 10 µl of a fresh, 1:1 diluted interphase extract in XB for 20 (lanes 5, 7 and 9) or 40 (lanes 6, 8 and 10) min at room temperature. At the respective times, each (both treated and untreated) aliquot of beads was washed in 0.5 ml XB and assayed for H1 kinase activity. (C) A mitotic extract that has been depleted of the cdc2/cyclin complex is rapidly converted to an interphase extract. 30 µl of interphase extract was activated with 30 µl of 100 nM GST-cyclin B. After 30 min at room temperature, each 10 µl aliquot was mixed with 10 µl of glutathione agarose beads, and incubated at room temperature for 15 min to allow binding of the active complex to the beads. The unbound mitotic extract in each aliquot was separated from its bead bound active complex for 2 (lane 3), 5 (lane 4), 10 (lane 5) or 15 min (lane 6) at room temperature, then added back to the beads. After 15 min , the beads were washed as described in (A) and assayed for H1 kinase activity. Lane 1 shows the H1 kinase activity of the untreated active complex, and lane 2 shows the H1 kinase activity of the active complex after isolation and incubation with a fresh interphase extract (diluted 1:1 with XB) for 15 min.

Figure 3-1

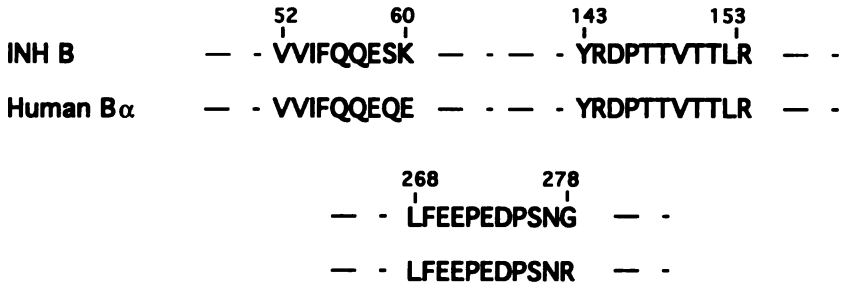

52
60
143
153
INH B — - VVIFQQESK — - — - YRDPTTVTTLR — -
Human Bα — - VVIFQQEQE — - — - YRDPTTVTTLR — -
268
278
— - LFEEPEDPSNG — -
— - LFEEPEDPSNR — -

Figure 3-2

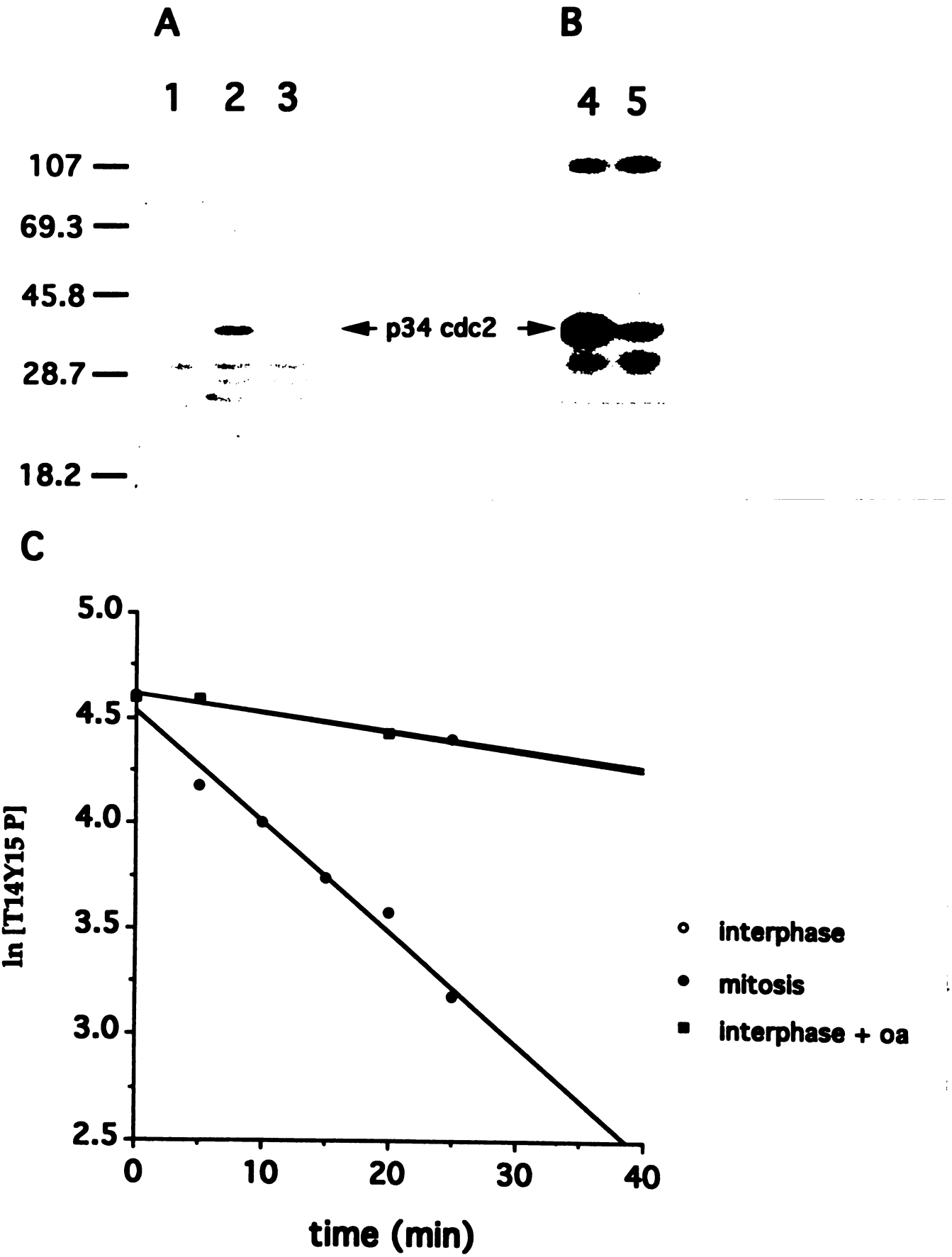

A
B
1 2 3
4 5
107
69.3
45.8
28.7
18.2
p34 cdc2
C
5.0
4.5
4.0
3.5
3.0
2.5
ln [T14Y15 P]
0
10
20
30
40
time (min)
interphase
mitosis
interphase + oa

Figure 3-3

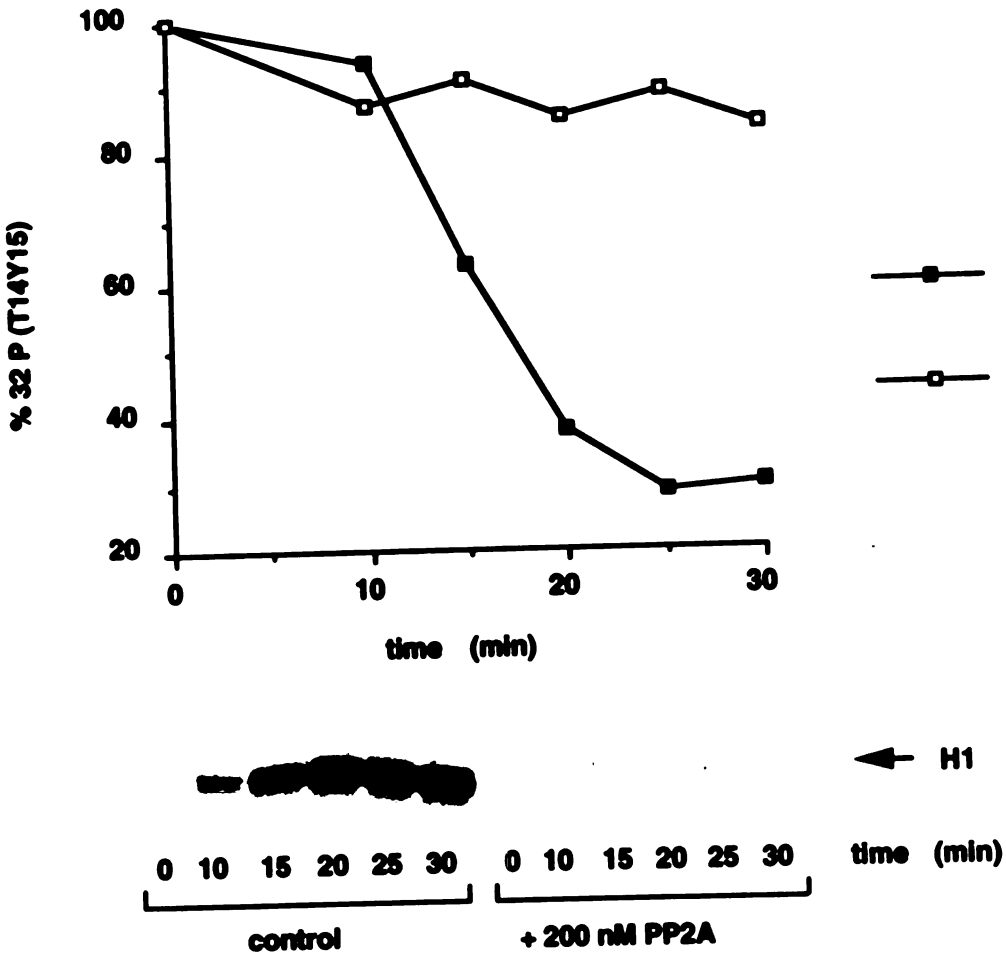

100
80
60
40
20
% 32 P (T14Y15)
0
10
20
30
time (min)
control
+ 200 nM PP2A
H1
0 10 15 20 25 30
0 10 15 20 25 30
time (min)
control
+ 200 nM PP2A

Figure 3-4

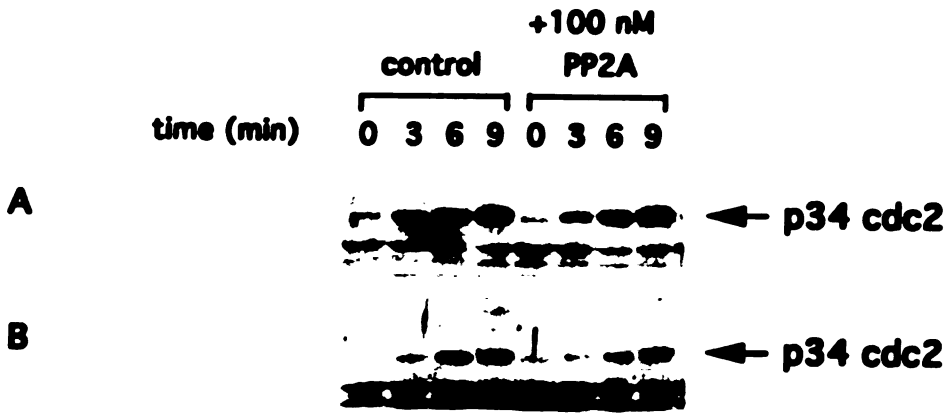


Figure 3-5

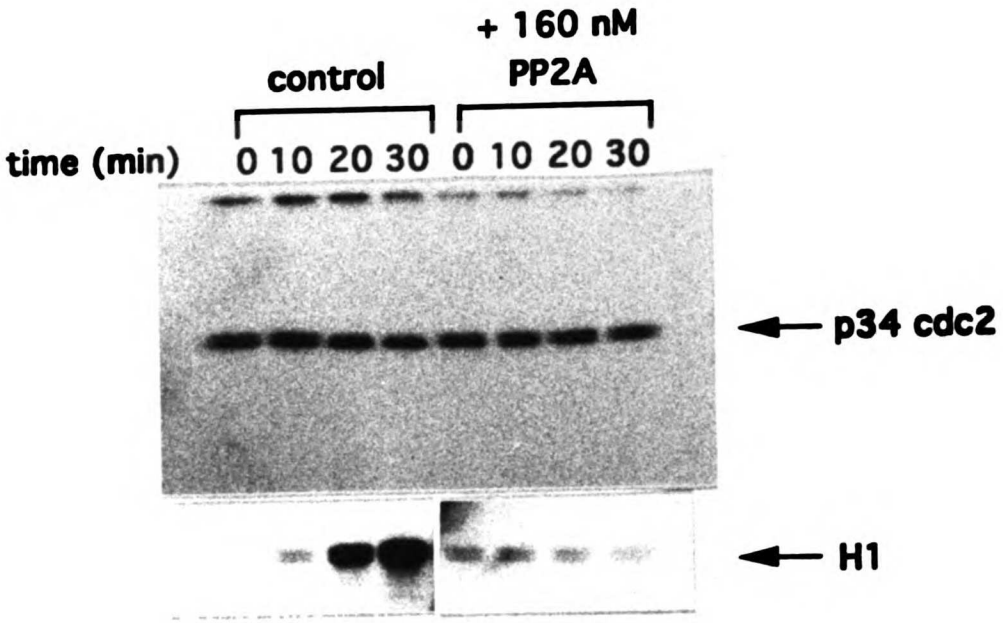


Figure 3-6A & B

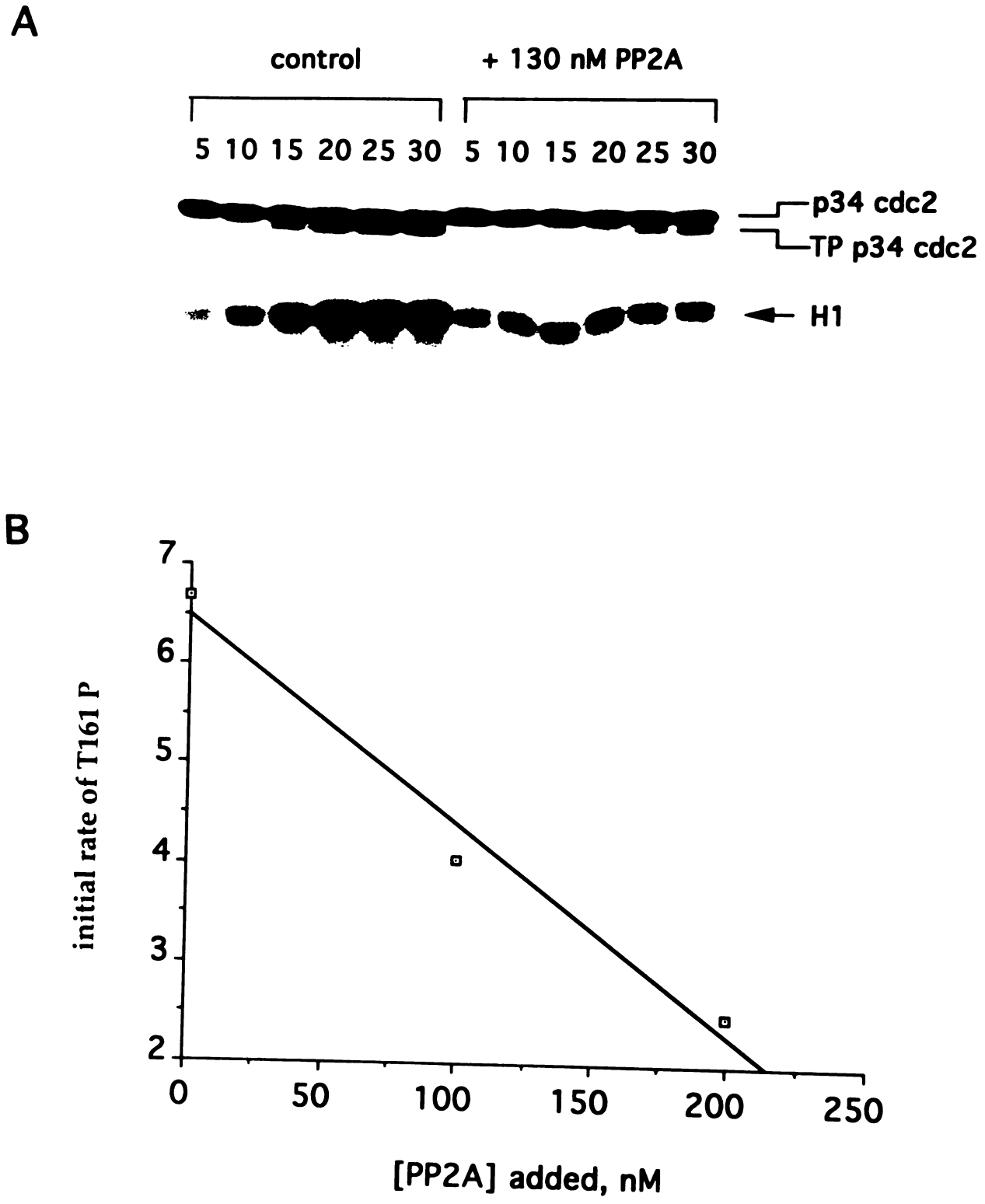
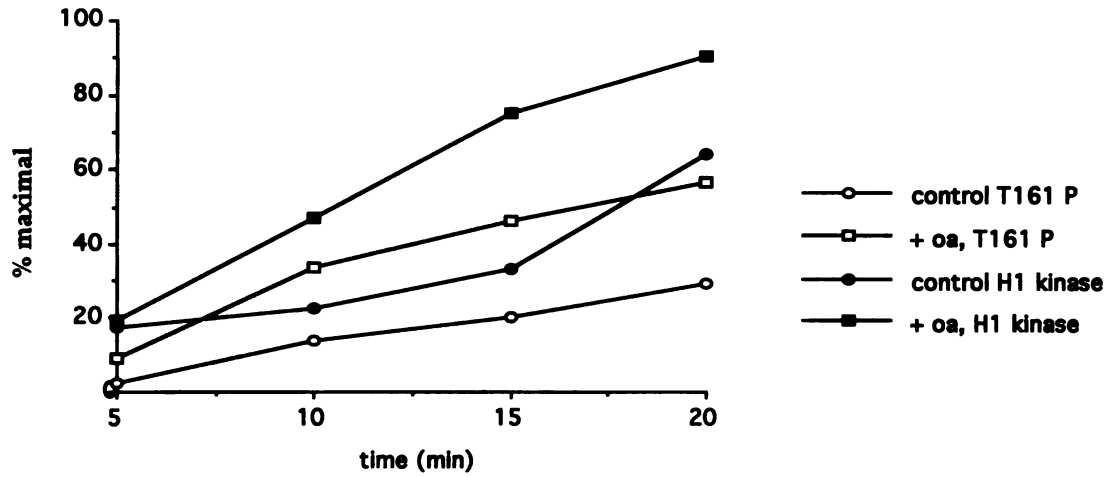

A
control
+ 130 nM PP2A
5 10 15 20 25 30 5 10 15 20 25 30
p34 cdc2
TP p34 cdc2
H1
B
initial rate of T161 P
7
6
5
4
3
2
0
50
100
150
200
250
[PP2A] added, nM

Figure 3-6C & D

C



D

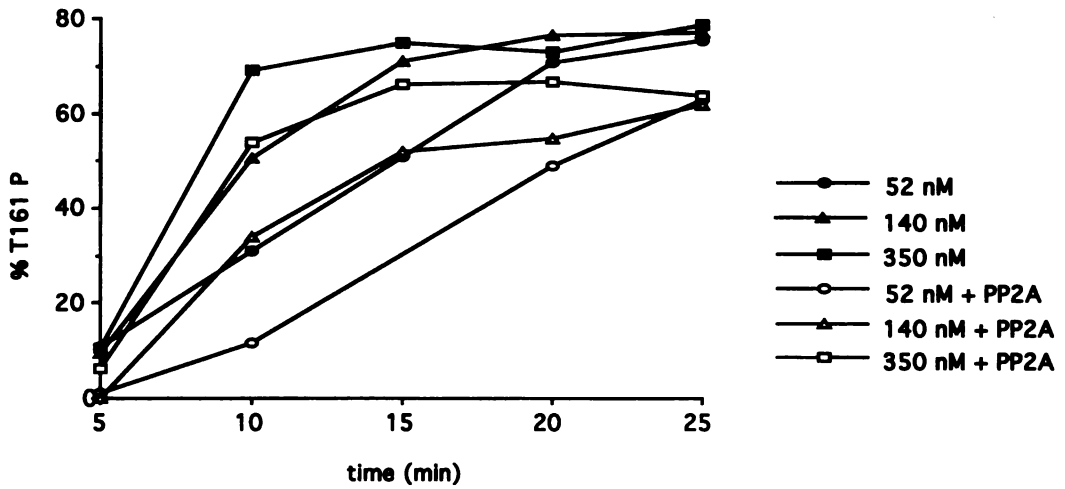


Figure 3-7

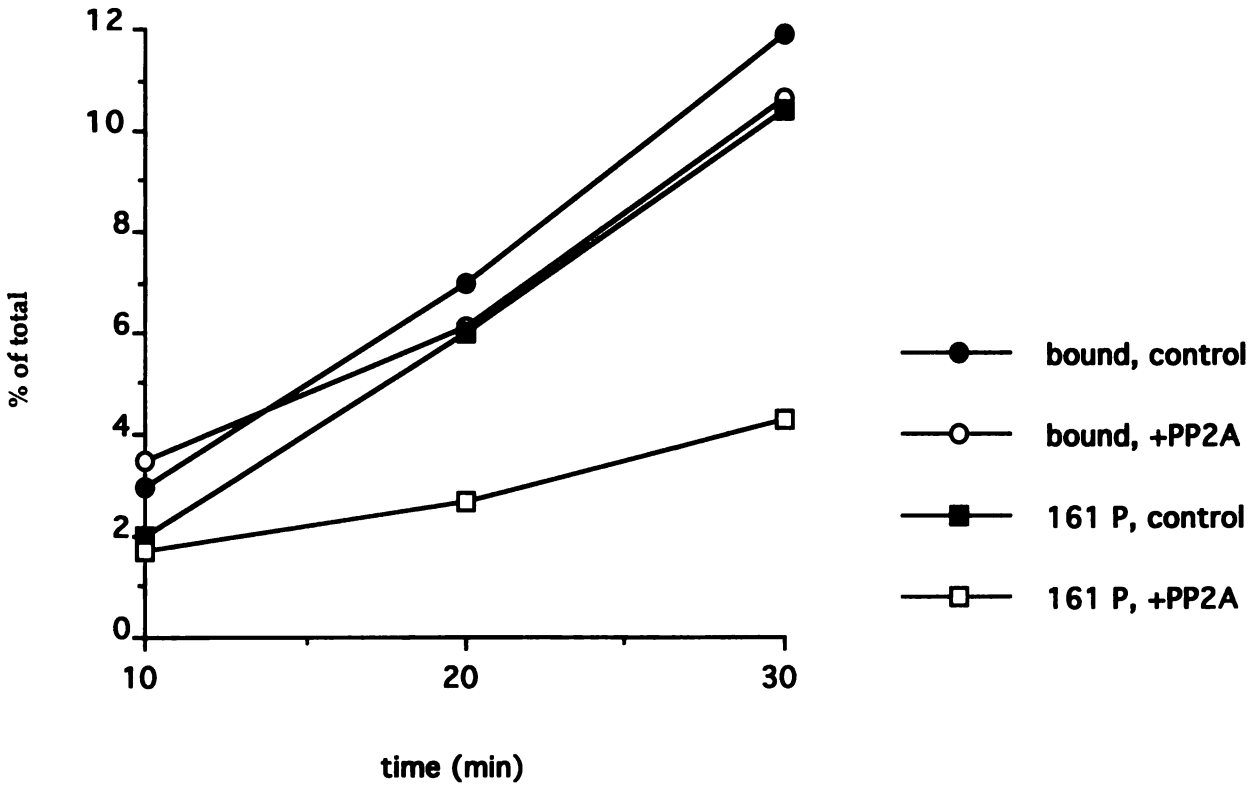

12
10
8
6
4
2
0
% of total
10
20
30
time (min)
bound, control
bound, +PP2A
161 P, control
161 P, +PP2A

Figure 3-8

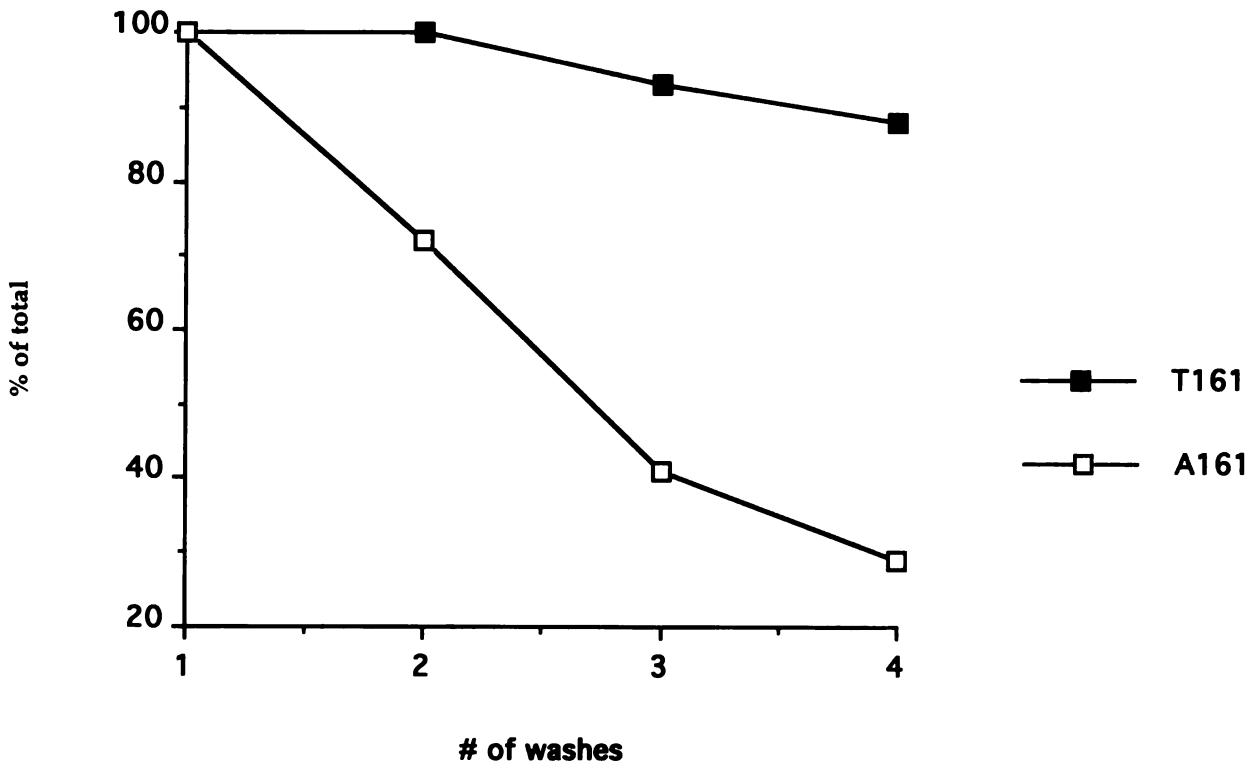


Figure 3-9

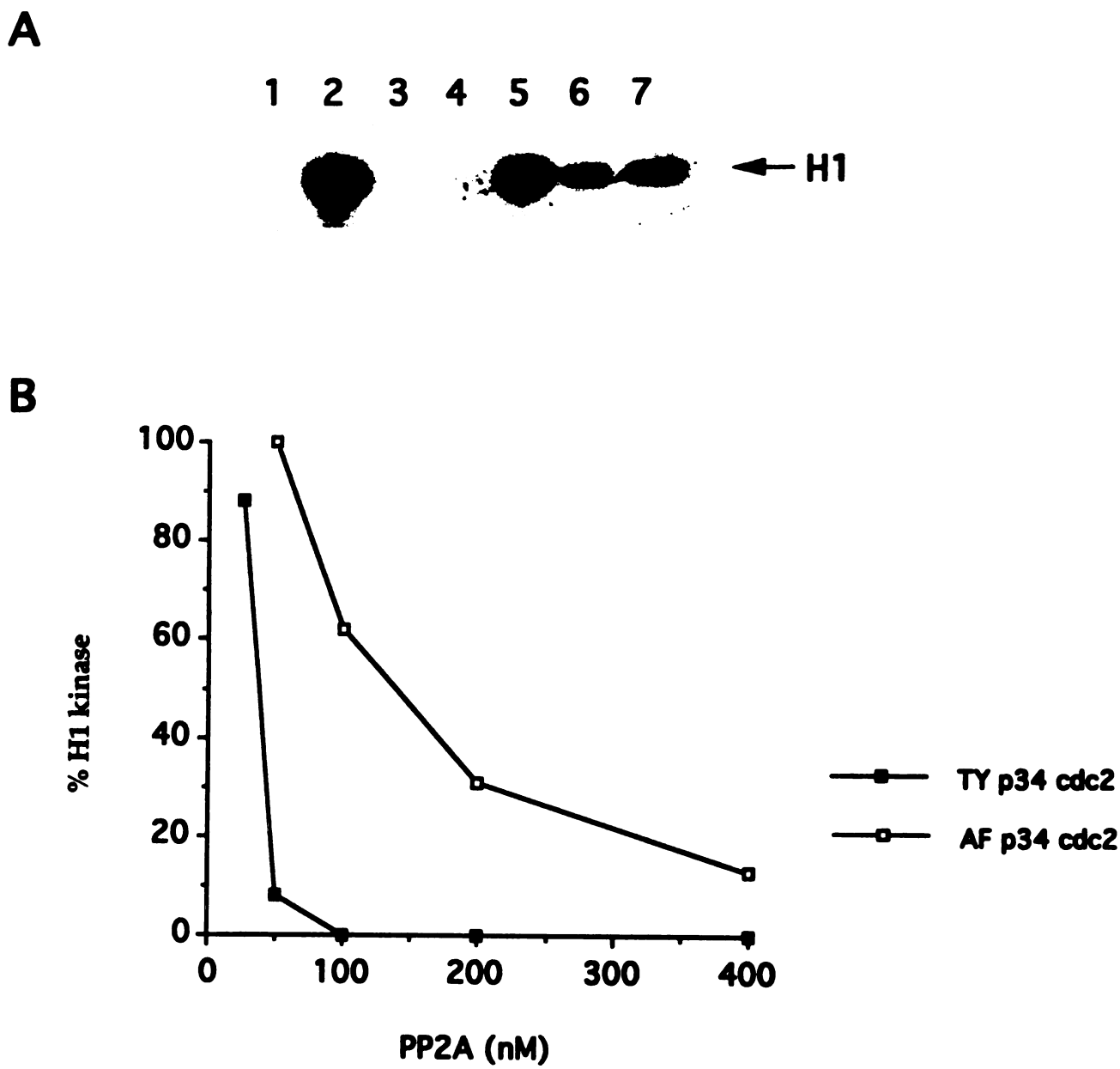
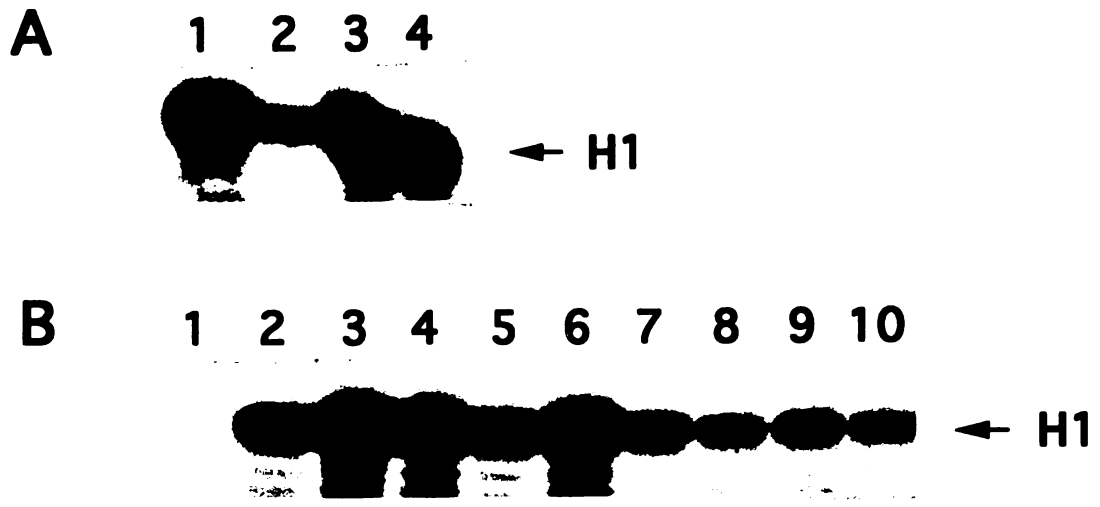

A
1 2 3 4 5 6 7
H1
B
% H1 kinase
100
80
60
40
20
0
0
100
200
300
400
PP2A (nM)
TY p34 cdc2
AF p34 cdc2

Figure 3-10



MATERIALS AND METHODS

Extracts and H1 kinase assays

Interphase extracts were prepared from eggs lain into MMR. After washing and dejellying, eggs were incubated in 100 μg/ml cycloheximide in MMR for 30 min prior to activation with the calcium ionophore A23187 (2μg/ml) in MMR. Cycloheximide was included at 100 μg/ml in all subsequent buffers. 10-20 min following activation, the eggs were washed in MMR, followed by washes in XB (100 mM KCl, 1 mM $MgCl_2$, .1 mM $CaCl_2$, 50 mM sucrose, 10 mM HEPES, pH 7.7), and finally into XB + protease inhibitors (10 μg/ml each of pepstatin, leupeptin and chymostatin). All subsequent steps were carried out exactly as previously described (Murray *et al.*, 1989; Solomon *et al.*, 1990) and extracts were stored in aliquots at -80°C. All of the activation reactions were carried out at room temperature. H1 kinase assays were performed by adding 6 ml of H1-ATP mix (200 μM ATP, 0.5mci/ml ^{32}P-γ-ATP and 500 μg/ml histone H1) to 10 μl beads or 12 μl of reaction mixtures diluted 1:12 into EB (80 mM b-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, pH 7.3). 1 μM okadaic acid was added to the ^{32}P-ATP-H1 mix in order to prevent dephosphorylation of H1 by PP2A during the kinase assay. Assays were carried out at room temperature for 5-10 min (within the linear range).

Proteins and Reagents

Okadaic acid was purchased from Calbiochem. Expression of wild type and mutant HA epitope-tagged*Xenopus* p34 ^{cdc2} in reticulocyte lysates has been described (Gautier *et al.*, 1991; Solomon *et al.*, 1992). The 12CA5 ascites (against the HA epitope) was generated at UCSF (Jim Berkhart) with

permission from Ian Wilson at the Scripps Clinic, San Diego. Immunoprecipitations using the ascites were performed essentially as described (Solomon *et al.*, 1992). The glutathione S-transferase cyclin B1 fusion construct containing an N-terminal fusion of glutathione S-transferase to *Xenopus* B1 cyclin, was cloned into the EcoR1 site of pGEX-1, and kindly provided to us by D. Kellog at UCSF, and the expression and purification of the fusion protein followed their procedure (Kellogg, D. and Murray, A., manuscript submitted). The purified*Drosophila* cdc25 protein, generously provided by Y. Gu and D. Morgan at UCSF, was expressed in *E.coli*, purified from inclusion bodies, and renatured as described in Gautier, *et al.* (1991). ^{32}P-γ-ATP (150 mci/ml, crude) used in the labelling reactions was purchased from NEN. Free glutathione and glutathione agarose beads were from Sigma. The beads were collected for washing typically by spinning in a microcentrifuge at 14,000 x g for ~5 seconds.

Partial purification of INH

INH was purified from egg extracts arrested in interphase. The initial steps of the purification, up to the first DEAE step were carried out as in Solomon *et al.* (1993). M. Solomon most kindly provided us with 500 mgs of the 150 mM-1M NaCl eluate from his DEAE column. The eluate was concentrated with ammonium sulfate, resuspended in buffer A (20 mM Tris, pH 8.0, 7 mM EGTA, 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 1mM DTT and protease inhibitors as described in Lee *et al.*, (1991), and rerun on a second 100 ml DEAE sepharose column, equilibrated in the same buffer. Bound proteins were eluted with a 500 ml 50-500 mM NaCl gradient. INH activity, assayed as described in Lee *et al.* (1991), eluted between 240-300 mM NaCl. Peak fractions were precipitated with 80% ammonium sulfate and the resulting precipitates

were collected in a 30 min spin at 10 K rpm in a JA 20 rotor. The pellets were resuspended in buffer A, adjusted to 1M ammonium sulfate and loaded onto a 10 ml tyrosine agarose column. Bound proteins were eluted with a 400 ml 1M-0M ammonium sulfate gradient. INH activity eluted between 550 and 450 mM ammonium sulfate. Peak fractions were concentrated by ammonium sulfate precipitation as described above and dialyzed against XB + 1 mM DTT + protease inhibitors (10μg/ml each of pepstatin, leupeptin, and chymostatin). The tyrosine agarose step yielded an ~30 fold purification over the DEAE step (see Lee *et al.*, 1991). The material use for the experiments in this paper was at least 1000 fold purified.

Microsequencing

For final purification, the protein mixture in the tyrosine agarose fractions containing the peak of INH activity was resolved further by a 5-20% sucrose density gradient. The peak INH fractions were subjected to SDS-PAGE (10%) and proteins were visualized by a brief Coomassie blue staining. The major band corresponding to the ~54 kD B subunit was recovered by electroelution, precipitated with acetone and redissolved in 0.1 M NH_4HCO_3. Cleavage of the protein was then carried out by the addition of 1 mg trypsin and incubation at 37°C for 12 hours. Tryptic cleavage fragments were separated on a C_{18} reversed phase column and selected peaks subjected to protein sequence analysis with an Applied Biosystems Sequencer, Model 475A (Applied Biosystems, Foster City, CA).

Generation of the T161 phosphorylated cdc2/cyclin complex

CAK activity, purified over 1800 fold, as described in Solomon *et al.*, (1993) and generously provided by M. Solomon, was used to phosphorylate

reticulocyte-translated cdc2 (as described in Solomon *et al.*, 1992). Briefly, 134 µl of reticulocyte lysate cdc2, containing the lys 33 to arg 33 mutation at ~ 10 ng/µl, was mixed with 15 µl of a 50 µg/ml solution of CAK (purified over DEAE, ammonium sulfate, hydroxyapatite, and mono Q, see Solomon *et al.*, 1993) in the presence of 134 µl ^{32}P-g-ATP (150 mci/ml, NEN), 15 µl of GST-cyclin B (50 nM, final concentration), and 30 µl of 10x XB (100 mM Hepes, pH 7.7, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 880 mM KCl). After incubation at room temperature for 30 min, the material was mixed with 300 µl of glutathione agarose beads for 20 min at RT, with rotation. After binding, the beads were washed extensively with XB^{++} (XB + 0.5 M NaCl + 0.1% NP40) + 1 mM DTT until the washes were free of counts (~10x15mls), followed by ~2x15ml washes in XB + 1 mM DTT. The complex was then eluted with 2 mls of XB containing 5 mM free glutathione + 0.1 mg/ml BSA + 1 mM DTT. The eluate was concentrated to 40 µl in a C-30 amicon micro-concentrator.

Generation of the Y15 phosphorylated cdc2/cyclin complex

cdc2/cyclin complexes labelled only on the inhibitory phosphorylation sites (Y15 and most likely also T14) were generated in a two stage reaction. In the first stage, 18 µl of interphase extract was diluted to 60 µl with XB containing 50 nM GST-cyclin B (final concentration). After incubation for 30 min at room temperature, the activated complexes, phosphorylated on T161 (Solomon *et al.*, 1990) were bound to 60 µl of glutathione agarose beads for 30 min at room temperature with rotation, followed by 2 washes in 15 mls of XB^{++} + 1 mM DTT, and 2 washes in 15 mls of XB + 1 mM DTT. The bead bound, active complexes were labelled on the inhibitory sites by a second stage incubation with 36 µl of interphase extract + 67 ml of ^{32}P-γ-ATP (150 mci/ml, NEN) + 12 µl of 10x XB. After extensive washing, the labelled, inactivated

complexes were eluted and concentrated to 50 µl exactly as described above for the ^{32}P-T161 phosphorylated substrate. In addition, we confirmed the 34 kD labelled protein to be cdc2 by demonstrating its high affinity to p13 ^{suc1} beads (Solomon *et al.*, 1990).

Gel electrophoresis and Immunoblotting

SDS-polyacrylamide gel electrophoresis for all of the experiments (except the T161 phosphorylation assay) were performed on 5-15% gradient gels. Separation of the T161 phosphorylated species from unphosphorylated p34 ^{cdc2} was performed on 10% polyacrylamide gels. An LKB semi-dry blotting apparatus was used to transfer proteins onto 0.22 µm nitrocellulose membranes (0.9 milliamp hr/cm^2). The anti-cdc2 blot was performed using antiserum generated against a *Xenopus* cdc2 C-terminal peptide, diluted 1:200. Antibody incubations were in 5% BSA + 0.1% TWEEN in 10 mM Tris pH 8.0, 150 mM NaCl; washes were in Tris/NaCl. Anti-phosphotyrosine blots were performed in the continuous presence of 5% nonfat dry milk + 0.1% TWEEN, in 10 mM Tris pH 8.0, 150 mM NaCl. 500 mM NaCl was included in the washes following the primary antibody (4G10 at 1 µg/ml, UBI) incubation. HRP-conjugated goat anti-mouse antibody (Cappel) was used at a 1:10,000 dilution. Immunodetection was performed using an enhanced chemiluminescence (ECL) system (Amersham). 2-fold dilutions of tyrosine phosphorylated p34 ^{cdc2} were used to assess the linear range for the detection system.

ACKNOWLEDGMENTS

We thank Mark Solomon for his generous contribution of purified CAK and DEAE eluates from which INH was purified. We also thank Marc Mumby for his helpful suggestions and generosity in sharing antibodies against PP2A, Doug Kellog for sharing his GST-*Xenopus* cyclin B1 construct, and Yong Gu for providing us with cdc25 protein. Discussions with Jian Kuang played an important role in this work. We also thank Peter Jackson, Jian Kuang and Andrew Murray for careful reading of the manuscript. This work was supported by the National Institute of General Medical Sciences and the Program of Excellence in Molecular Biology of the National Heart Lung and Blood Institute.

Chapter Four

Perspectives and Conclusions

Inhibition of threonine 161 phosphorylation on cdc2 by INH

INH was identified as an endogenous inhibitor of M phase induction in Xenopus oocytes and eggs. Since cdc2 was shown to regulate entry into M phase, it seemed likely that INH might prevent M phase induction by inhibiting the activation of cdc2. The observation that INH inhibits the premature activation of cdc2 predicted that INH would regulate an event(s) that triggers the activation of cdc2. With the aim of identifying the triggering event, we examined the reaction pathways that determine cdc2 activity for the INH-sensitive pathway. Our results indicate that neither the pathway regulating tyrosine 15 phosphorylation nor tyrosine 15 dephosphorylation is affected directly by INH. In fact, the only pathway regulated directly by INH appears to be that affecting threonine 161 phosphorylation. This presented an apparent paradox, since INH affects somehow the switch in tyrosine kinase and tyrosine phosphatase activities that accompany the transition. In order to reconcile these data, we proposed a model in which INH, by inhibiting the phosphorylation of threonine 161 on cdc2 during the pre-transition phase, inhibits indirectly the switch in the balance between tyrosine kinase and tyrosine phosphatase activities that is required for the final activation of cdc2. By delaying the phosphorylation of threonine 161, INH would inhibit the accumulation of the active cdc2/cyclin B complex during the pre-transition phase, that has been proposed to initiate an autocatalytic activation loop. This positive feedback loop model places the phosphorylation of threonine 161 upstream of the transition and more central to the trigger for mitotic initiation. As will be discussed later, we now have evidence that this model cannot by itself account for the role of INH in

inhibiting the trigger.

Although we do not have direct evidence for the involvement of the threonine 161 phosphorylation reaction in the mitotic trigger, it seems that the regulation of threonine 161 phosphorylation by INH could provide a mechanism for regulating cdc2 activation, either upstream of the tyrosine phosphorylation/dephosphorylation pathway, or on its own when the inhibitory tyrosine phosphorylation pathway is inoperative, e.g., when a lengthy lag phase preceding mitosis is not required, such as in the second to twelfth rapid cleavages of the Xenopus embryo. Ferrell et al. (1991) have found that in these rapid cell cycles without a G2 phase, cdc2 is not tyrosine-phosphorylated, and yet MPF activity oscillates. It is possible that here, the activation of cdc2 is limited by threonine 161 phosphorylation (although it could also be limited by the accumulation of cyclin). In a situation when a lengthy lag phase *does* precede mitosis (such as in the first embryonic cell cycle), the regulation of threonine 161 phosphorylation could be harnessed to indirectly regulate, at least in part, the tyrosine phosphorylation/dephosphorylation pathway.

How INH regulates the threonine 161 phosphorylation of cdc2 should be explored. Does it inhibit the kinase CAK directly or indirectly? The threonine 161 phosphorylation reaction appears to be stimulated by cyclin, but whether this effect is due to cyclin activating CAK independently of its role as a substrate for CAK remains to be determined. There is some intriguing preliminary data suggesting the involvement of certain MPM-2 antigens in the upstream regulation of threonine 161 phosphorylation. MPM-2 antigens are defined by a monoclonal antibody that recognizes a set of ~40 mitosis-specific phosphoproteins. A consensus sequence for a phosphoacceptor site that generates MPM-2 antigenicity when phosphorylated has been deduced

recently by the screening of peptide expression libraries. The site is somewhat similar to the site for cdc2 kinase (both are proline-directed), but is clearly distinct. MPM-2 antigens have been shown to be involved in the amplification of MPF (Kuang et al., 1989); MPM-2 antibodies delay the activation of cdc2 kinase and MPF in Xenopus extracts, although they do not cross react with cdc2 kinase (Kuang et al., 1991). A preliminary experiment by Kuang et al. (unpublished) shows that the addition of MPM-2 antibodies to Xenopus interphase extracts delays the phosphorylation of cdc2 on threonine 161, suggesting that this reaction might be regulated by an MPM-2 antigen. Furthermore, Kuang et al. (manuscript in preparation) have demonstrated the existence of three factors (one of them is cdc25) in Xenopus egg extracts that can activate the latent cdc2 kinase through dephosphorylation of the inhibitory site(s). All three factors are MPM-2 antigens. Thus it is tempting to speculate that the kinase(s) (not yet identified) that phosphorylates the MPM-2 epitope affects the threonine 161 phosphorylation rate, as well as the rate of phosphorylation and dephosphorylation of the inhibitory site(s).

Is threonine 161 phosphorylation on cdc2 the only reaction regulated by INH?

The model placing the phosphorylation of threonine 161 upstream of the switch in tyrosine kinase and tyrosine phosphatase activities fit nicely at first with the positive feedback loop model described above, initially proposed by Solomon et al., in which the accumulation of a low level of active cdc2 during the pre-transition phase triggers the switch in the balance between tyrosine kinase and phosphatase activites. Preliminary experiments to test this model yielded a surprising result, that the only known end product of cyclin B addition, the cdc2/cyclin B complex, cannot

drive interphase Xenopus extracts into mitosis. It appears, instead, that the formation of active cdc2/cyclin complexes is only one of the cyclin-initiated reaction pathways required for triggering the transition. At least one additional pathway, as yet uncharacterized, that leads to the activation of the tyrosine phosphatase and the inactivation of the tyrosine kinase appears to be required for the stable activation of cdc2. This pathway, like T161 phosphorylation and the formation of active cdc2/cyclin complexes, seems to be stimulated by cyclin and inhibited by INH, as the requirement for this pathway is bypassed by either the addition of free cyclin or by the addition of okadaic acid simultaneously with the active cdc2/cyclin complex. We must conclude, then, that although threonine 161 phosphorylation may be an important regulatory step in the trigger, there exists at least one additional target of INH important for the induction of M phase. This observation implies that INH might negatively regulate more than one pathway during M phase initiation. Given the involvement of MPM-2 antigens in the activation of cdc2 through tyrosine dephosphorylation, and the potential involvement of MPM-2 antigens in controlling threonine 161 phosphorylation, an intriguing possibility is that INH might regulate the kinase(s) that phosphorylates the MPM-2 antigens and thereby inhibit both the pathway(s) controlling tyrosine phosphorylation/dephosphorylation and that regulating threonine 161 phosphorylation.

The existence of more than one pathway regulating entry into mitosis has been suggested previously by researchers in other systems. First, in A. nidulans, mutations in nimA lead to cell cycle arrest in G2. Surprisingly, the activity of p34 ^{cdc2} is high, suggesting that activation of p34 ^{cdc2} is not sufficient to trigger entry into mitosis (Osmani et al., 1991). Second, transient expression of a constitutively active form of calcium/calmodulin

dependent protein kinase II in a mammalian cultured cell line leads to cell cycle arrest in G2. During the G2 block, cells accumulate high levels of p34 ^{cdc2} kinase activity, again suggesting that other changes in protein phosphorylation, besides those involved in p34 ^{cdc2} activation, may be necessary for mitotic induction (Planas-Silva and Means, 1992). These examples not only suggest the insufficiency of active p34 ^{cdc2} in inducing entry into mitosis, but also indicate that active p34 ^{cdc2} can be maintained stably in a premitotic cell. While our results suggest the involvement of multiple pathways in the activation of p34 ^{cdc2} itself, these studies suggest the existence of multiple pathways, in addition to those directly regulating p34 ^{cdc2}, for the induction of mitosis. Whether or not INH plays any role in regulating these putative 'extra-p34 ^{cdc2}' pathways is completely unknown, and the state of MPM-2 phosphorylation in these G2 arrests has not been shown definitively.

Identifying the target of INH

preMPF

INH was originally defined and purified as an inhibitor of preMPF activation. But with the emergence of a large body of information on the mechanics of cdc2 activation in interphase extracts by cyclin protein, we chose this as the system in which to identify the target of INH. Unfortunately, the results leave us short of identifying the target, and we are left with an incomplete picture of the mechanism of MPF activation. In hindsight, a more natural course might have been the identification of preMPF through its purification, since the target of INH, by definition, resides in preMPF. Although Cyert and Kirschner began to characterize preMPF, the

molecular identity of preMPF remains unknown. Given the available information, preMPF is unlikely to correspond simply to the latent cdc2/cyclin B complex in oocytes. The preMPF activity occurred in at least two peaks by gel filtration chromatography, estimated at ~700 kD and ~400 kD. Furthermore, the mixing of side fractions containing no detectable preMPF activity resulted in the reconstitution of additional activity, suggesting that there might be multiple components in preMPF. This speculation is similar to what has been posed already for MPF by Kuang et al. (discussed above), and underscores the importance of studying MPF as a system of interdependent pathways. The advantage in pursuing preMPF is that the activity does not require stabilization, as it is in the latent form until activation by ATP addition. Therefore, the system may be more amenable to studies examining the interdependency of factors involved in the conversion of preMPF to MPF.

MPM-2 phosphorylation

An intriguing possibility, as described above, is that INH somehow regulates the kinase that phosphorylates the MPM-2 antigens that are involved in the final activation of cdc2. Consistent with this proposal is the observation that addition of purified INH to an interphase extract and cyclin delays the appearance of MPM-2 antigens during the pre-transition phase (unpublished results). That elevated levels of INH block the burst of MPM-2 phosphorylations normally accompanying the final activation of cdc2 is expected. However, the inhibition of MPM-2 phosphorylations during the lag by INH suggests that a physiological target of INH might be either the kinase that phosphorylates the MPM-2 epitope, or an upstream regulator of the MPM-2 kinase. The kinase that phosphorylates the majority of MPM-2 antigens has not yet been identified. However, preliminary fractionation of

an M-phase extract for an activity that confers immunoreactivity with MPM-2 on interphase extract proteins immobilized on nitrocellulose has yielded a single peak of activity (Kuang et al., unpublished results), suggesting that there might be a single kinase responsible for the majority of MPM-2 phosphorylations; further fractionation will be required to settle this point. Whether INH inactivates this kinase(s) can be tested directly once this kinase(s) has been partially purified. As an aside, the peak of MPM-2 kinase activity is found in the void volume on gel filtration columns (~700 kD), reminiscent of the behavior of preMPF during gel filtration chromatography. Naturally, this leads to the speculation that preMPF corresponds, at least in part, to the MPM-2 kinase. The only available data that is consistent with this notion is that the MPM-2 kinase(s) in interphase extracts can be activated in the absence of cyclin, if the extract is prepared in EB plus okadaic acid, effectively the conditions that lead to the activation of preMPF.

Although there is evidence for the involvement of MPM-2 antigens during cdc2 activation and in addition, evidence that certain MPM-2 antigens are required for the maintenance of the mitotic state, it is not clear whether the MPM-2 phosphorylation itself is mandatory for M-phase induction. The formal possibility that MPM-2 phosphorylations occur fortuitously on important regulators of cdc2 with no consequence on their relative activities still exists. Determining whether MPM-2 phosphorylation, per se, is a key event during M-phase induction will require the identification of the MPM-2 antigens in question and their sites of phosphorylation. Nevertheless, the available information strongly predicts that the MPM-2 kinase is an important regulator of M-phase induction, and that INH regulates the MPM-2 kinase either directly or indirectly.

Cyclin

So far, two general approaches to the identification of the physiological target of INH have been discussed: The identification of preMPF, that by definition contains the INH target; and the upstream regulation of threonine 161 phosphorylation, potentially involving an MPM-2 antigen(s). The final approach to be discussed is the characterization of the initial events that occur upon cyclin addition that ultimately lead to the stable activation of cdc2.

As described above, while the addition of cyclin induces all of the events necessary for the generation of a stable mitotic state, the addition of the only known end product of cyclin, the cdc2/cyclin kinase complex, is insufficient for bringing about the shift in the balance between tyrosine phosphorylation and tyrosine dephosphorylation that is necessary for the generation of the mitotic state. This implies that cyclin induces another limiting pathway(s), required to stabilize cdc2 in the active conformation. That okadaic acid addition bypasses the requirement for this pathway(s) suggests that INH negatively regulates this pathway, and this points to the third potential target of INH.

The observation that cyclin, after the mitotic transition, is unable to initiate the proposed pathway(s), suggests that this function of cyclin is transient, peaking sometime during the pre-transition phase, prior to the final activation of cdc2. The most direct approach towards characterizing the proposed pathway would begin with the isolation of an intermediate activity induced by cyclin addition to interphase extracts, that could stabilize the known end product of cyclin addition, i.e., the activated cdc2/cyclin complex when added to an interphase extract. This might be a difficult task, since the proposed activity appears to occur transiently after cyclin addition, declining

during the interphase to mitotic transition. Defining conditions that stabilize the proposed intermediate would clearly facilitate the task. One way to stabilize the intermediate might be to prevent the final activation of cdc2. It is possible, for example, that addition of subthreshold levels of cyclin to interphase extracts would lead to the accumulation of the intermediate, be it the unphosphorylated cdc2/cyclin complex or some other complex containing cyclin. On the other hand, accumulation of the active intermediate may require a threshold level of cyclin. The nature of the cyclin threshold remains unknown. At this point, the cyclin threshold represents the level of cyclin that is required to counteract the effect of INH. If the rate limiting event, stimulated by cyclin and inhibited by INH, is the formation of the intermediate, then the intermediate may not be present in the active state at subthreshold levels of cyclin. If this is the case, the only viable alternative may be to isolate the intermediate from the pre-transition phase of an activation reaction.

SUMMARY

Some general approaches towards the identification of the target of INH have been outlined here. It is possible that all of the approaches will lead to the same point eventually, especially if there exists a single target of INH that is crucial for preventing the premature initiation of M-phase. Working upstream of the threonine 161 phosphorylation reaction may in addition reveal the events occurring downstream of the target of INH, and examining the initial events that occur upon cyclin addition may reveal the events leading up to the target of INH. It is not clear what parts of the pathway(s) will be unmasked by studying preMPF. Each approach is likely to

lead to the identification of additional components in the network of enzymes controlling M-phase induction. The use of INH as a probe for the mitotic trigger has shown us that our picture of MPF activation is incomplete, but this in itself is an important finding. That mitotic induction is not so simple is a satisfying thought, and promises a store of surprises awaiting us in future studies.

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