Biochemical Characterization of the p34<sup>cdc2</sup> Protein Kinase Component of Purified Maturation-promoting Factor from Xenopus Eggs

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Genetic studies in the fission yeast Schizosaccharomyces pombe and biochemical data in oocytes and eggs of Xenopus laevis have implicated the product of the cdc2<sup>+</sup> gene as critical for the G2 to M transition in the cell cycle. The product of the cdc2<sup>+</sup> gene is a 34-kDa serine/threonine protein kinase, designated p34<sup>cdc2</sup>, that is a component of purified maturation-promoting factor (MPF) and also of purified mammalian growth-associated histone H1 kinase. The biochemical properties of p34<sup>cdc2</sup> H1 kinase activity in the MPF complex were studied. Phosphorylation of the p45<sup>MPF</sup> component in the MPF complex by p34<sup>cdc2</sup> exhibited kinetics consistent with an intramolecular reaction. On gel-serial gradient centrifugation, MPF kinase against several substrates sedimented with an apparent Mr = 45,000–55,000. p34<sup>cdc2</sup> was found to utilize ATP, GTP, and adenosine 5'-O-(3-thiotriphosphate) with apparent Km values of 75, 700, and 250 μM, respectively. The kinase activity was inhibited by β-glycerophosphate, NaF, and zinc, whereas ρ-nitrophenyl phosphate was slightly stimulatory. The relative rates of phosphorylation of various substrates by MPF and growth-associated H1 kinase were similar. These findings should prove useful in further work on the regulation of MPF kinase activity and characterization of its substrates.

Recent years have seen remarkable advances in our understanding of the molecular basis of cell cycle control in mitosis (M-phase). New insights have come primarily from genetic analysis in the fission yeast Schizosaccharomyces pombe (1) and from biochemical analysis in oocytes and eggs of the frog, Xenopus laevis (2). In S. pombe, a substantial body of evidence has accumulated to indicate that the product of the cdc2<sup>+</sup> gene is critically involved in the G2 to M transition. The product of the cdc2<sup>+</sup> gene is a 34-kDa serine/threonine protein kinase, designated p34<sup>cdc2</sup>, that is regulated in a complex fashion by a number of other cell-cycle components, several of which are also protein kinases (3–7). A human homolog of cdc2<sup>+</sup> has been cloned from a HeLa cell cDNA library by complementation analysis (8), suggesting that the role of p34<sup>cdc2</sup> is sufficiently conserved to support function of the human gene product in yeast.

In Xenopus, biochemical analysis of the cell cycle has been concerned largely with the characterization of a cytoplasmic activity known as maturation-promoting factor (MPF).<sup>1</sup> MPF was first described in 1971 as an activity in "phase cytoplasm of maturing Xenopus oocytes that could cause immature oocytes to undergo meiotic maturation without hormonal stimulation or protein synthesis (9, 10). Cell-cycle studies showed that MPF activity appears not only in Xenopus oocytes undergoing meiotic maturation but also in maturing oocytes of other species, and during mitosis in mammalian cells and the budding yeast Saccharomyces cerevisiae (11–15). Despite the apparent fundamental importance of MPF in the cell cycle, until recently little progress had been made in its purification, partly because the oocyte microinjection assay can detect MPF activity only in highly concentrated fractions (16) and because its activity is highly unstable.

Extracts of Rana eggs and Xenopus eggs containing MPF activity have been shown to cause isolated sperm chromatin to form chromosomes in vitro (17, 18). Moreover, the addition of partially purified MPF, which had been detected by the oocyte microinjection assay, caused nuclear envelope breakdown, chromosome condensation, and spindle formation in vitro (18, 19). The induction of these early mitotic events in vitro formed the basis of a new assay for MPF, which allowed the purification of Xenopus MPF to near homogeneity (20). Highly purified MPF, which can induce M-phase both in the cell-free system and in microinjected oocytes, consists of two proteins of Mr = 34,000 and 45,000. The final preparation expresses a protein kinase activity that can phosphorylate the 45-kDa component, H1 histone, protein phosphatase inhibitor 1, and α-casein (20).

It seemed likely that the 34-kDa component of MPF possessed the protein kinase activity because the 45-kDa component became phosphorylated only in fractions that also contained the 34-kDa component (20). Because of the similar molecular weight of this component of MPF and that of the cdc2<sup>+</sup> gene product, experiments were carried out to determine whether there was any relationship between these two proteins. These studies used antisera raised against a unique stretch of 16 amino acids, termed the PSTAIR sequence, that is perfectly conserved in p34<sup>cdc2</sup> and its homologs from yeast to man, but that is not present in any other members of the protein kinase family so far sequenced (8). This antibody immunoblotted the 34-kDa component of MPF, indicating that it is the product of a Xenopus homolog of the cdc2<sup>+</sup> gene (21). In addition, both components of MPF could be immunoprecipitated, indicating that the 45-kDa protein exists in a complex with the 34-kDa protein kinase (21). Moreover, immunoprecipitated Xenopus p34<sup>cdc2</sup> retains the ability to

<sup>1</sup>The abbreviations used are: MPF, maturation-promoting factor; ATP-S, adenosine 5'-O-(3-thiotriphosphate); MAP-2, microtubule-associated protein-2; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenbis(oxyethylenenitrito)]tetrasacetic acid; MES, 4-morpholineethanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine.
phosphorylate H1 histone (21). Studies of the phosphorylation state of p34\textsuperscript{cdc2} during the cell cycle have revealed that it is active when dephosphorylated and inactive when phosphorylated (22).

Recently, the 45-kDa component of MPF has been identified as a Xenopus B-type cyclin.\textsuperscript{2} Cyclins are proteins that accumulate continuously during interphase but that are quantitatively degraded at the metaphase/anaphase transition during mitosis (24). Cyclins B1 and B2 are very similar in sequence and both appear to be present in highly purified MPF.\textsuperscript{2} The phosphorylation of cyclins B1 and B2 by p34\textsuperscript{cdc2} may have functional consequences for MPF activity or stability, inasmuch as addition of ATP\textgamma{}S to extracts is essential for the purification of active MPF (20).

Previous findings in several laboratories have shown that in mammalian cells H1 becomes phosphorylated in M-phase at specific sites with a stoichiometry of 3 to 6 phosphates/molecule (25, 26). The protein kinase responsible, termed the growth-associated H1 kinase (27), has been purified recently from chromatin fractions of Novikoff hepatoma cells and found to include a 34-kDa protein.\textsuperscript{3} The PSTAIRE antibody can immunoblot the 34-kDa component of growth-associated H1 kinase and can immunoprecipitate the growth-associated H1 histone kinase activity (28). Moreover, the homologs of p34\textsuperscript{cdc2} from Xenopus, budding yeast, and fission yeast specifically phosphorylate the same sites in H1 histone as mammalian growth-associated H1 kinase. In contrast, extracts of budding yeast deficient in the CDC28 gene, a homolog of cdc2 (29), are unable to phosphorylate these sites (28). Together these studies suggest that the growth-associated H1 kinase from mammalian cells also contains a p34\textsuperscript{cdc2} homolog, and that p34\textsuperscript{cdc2} is a protein kinase that functions in M-phase control not only in yeasts, but also in Xenopus and mammalian cells. Although these four protein kinases, identified in yeast, frog, and rat, now appear to be the same or very closely related, little is known about their biochemical properties. We report here characterization of p34\textsuperscript{cdc2} kinase activity in the Xenopus MPF complex.

**EXPERIMENTAL PROCEDURES**

Materials—MPF was purified from unfertilized eggs of *X. laevis* as previously described (20) except that the active fractions from the Mono S column were not dialyzed before freezing and storage at −70 °C. [γ-\textsuperscript{32P}]ATP and [γ-\textsuperscript{32P}]GTP were prepared by the method of Johnson and Walseth (30). Adenosine 5'-[\textgamma-\textsuperscript{35S}]thiotriphosphate (ATP\textgamma{}S) were obtained from Boehringer Mannheim and α-casein was obtained from Sigma. Protein phosphatase inhibitor 1, microtubule-associated protein 2 (MAP-2), and partially purified growth-associated H1 kinase were generously provided by J. W. Johansen, University of Colorado Health Sciences Center, Denver, CO, T. W. Sturgill, University of Virginia, School of Medicine, Charlottesville, VA, and T. A. Langan, University of Colorado Health Sciences Center, Denver, CO, respectively.

Protein Kinase Assays—Except as noted in the legends to various figures, reactions were incubated for 30 min at 30 °C in a final volume of 30 μl containing 50 mM Hepes (pH 7.5), 15 mM MgCl\textsubscript{2}, 30 mM 2-mercaptoethanol, 100 μM [γ-\textsuperscript{32P}]ATP (1 to 5 cpm/μmol), 0.5 μg/ml H1 histone, 0.1 μg/ml bovine serum albumin, 0.01% Brij-35, and an appropriate amount of enzyme. Reactions were terminated by the addition of 15 μl of glacial acetic acid, and phosphorylated H1 was isolated on a P81 phosphocellulose paper (31). Radioactivity was quantified by counting Cerenkov radiation. Additional details are provided in Materials and Methods. The positions of molecular weight standards are indicated alcohol dehydrogenase (yeast, M\textsubscript{r} = 145,000), fraction 6; and peroxidase (horseradish, M\textsubscript{r} = 43,000), fraction 16. Sedimentation was from right to left, △, H1 histone; ●, MAP-2.

![FIG. 1. Sedimentation analysis of MPF kinase activity. A sample of MPF was centrifuged at 54,000 rpm for 20 h in a SW55 rotor at 3 °C through a linear glycerol gradient (10-30%) containing 50 mM β-glycerophosphate (pH 6.5), 5 mM MgCl\textsubscript{2}, 5 mM EGTA, 25 mM NaCl, 2 mM dithiothreitol, 0.01% Brij-35. Fractions were collected and samples were assayed for kinase activity as described under "Experimental Procedures." Recovery of kinase activity was almost 100%, and no activity could be recovered from the bottom of the tube. The positions of molecular weight standards are indicated: alcohol dehydrogenase (yeast, M\textsubscript{r} = 145,000), fraction 6; and peroxidase (horseradish, M\textsubscript{r} = 43,000), fraction 16. Sedimentation was from right to left. △, H1 histone; ●, MAP-2.](image1)

![FIG. 2. Effect of the concentration of MPF on phosphorylation of its p45\textsuperscript{cytin} component. Various amounts of MPF were incubated in the presence of [γ-\textsuperscript{32P}]ATP (30 cpm/μmol) for 90 s at 30 °C. Products of the reaction were resolved by polyacrylamide gel electrophoresis, and radioactivity in p45\textsuperscript{cytin} was determined by liquid scintillation spectrometry. Other experiments showed that incorporation of radiolabel into p45\textsuperscript{cytin} was linear for at least 120 s for 20 μl of MPF/reaction.](image2)
was present in concentrations from 10 to 150 µM. The same sedimentation behavior was seen with various MPF preparations regardless of where they had eluted from the TSK column, with or without further purification. The ionic conditions for the two procedures are similar, and the reason for this discrepancy in apparent M₅₀ is not clear. It should be noted, however, that the sedimentation behavior shown here is similar to that of the major peak of MPF activity, assayed by microinjection, when crude extracts were analyzed by sucrose gradient centrifugation (34). In other studies, partially purified MPF was reported to elute as a peak of 90–100 kDa from a Sephacryl S200 column (35). These results are most consistent with Xenopus MPF existing as a heterodimer, but further work is needed to establish the ratio of subunits in purified Xenopus MPF preparations. MPF isolated from starfish oocytes has been reported to exist as a heterodimer containing one molecule of p45αcyclin and one molecule of p40βcyclin (36).

Recently, it was found that highly purified MPF expressed kinase activity against MAP-2 also. Fig. 1 illustrates that MAP-2 kinase activity co-sedimented with H1 kinase activity. In addition, kinase activity against the 45-kDa protein, α-casein, and inhibitor 1 also co-sedimented with H1 kinase activity (data not shown). These results are consistent with the idea that there is only one kinase present in MPF and that the phosphorylation of the proteins other than H1 is not due to a contaminating kinase(s). Phosphoamino acids detected in the substrates phosphorylated by MPF were as follows: 45-kDa protein, H1, and MAP-2, phosphoserine and phosphothreonine; α-casein, phosphothreonine; inhibitor 1, phosphothreonine (data not shown).

As originally reported by Lohka et al. (20), incubation of purified MPF with [γ-32P]ATP led to phosphorylation of the p45αcyclin component. The kinase responsible was most likely p34αcyclin because the preparation was highly purified and phosphorylation of p45αcyclin occurred only in fractions that also contained p34αcyclin. Since both subunits of MPF exist together in a complex, it seems likely that p45αcyclin phosphorylation would occur by an intramolecular mechanism. To assess this possibility directly, the rate of phosphorylation of p40βcyclin in a purified MPF fraction was monitored as a function of MPF concentration in the assay. As shown in Fig. 2, the rate of the reaction increased only slightly over a 20-fold range of MPF concentrations. This result is consistent with p40βcyclin phosphorylation in the MPF complex occurring by an intramolecular mechanism. Other experiments have shown, however, that purified MPF can also phosphorylate exogenous, bacterially produced cyclin.

In other experiments the ability of highly purified MPF to use ATP, GTP, and ATPγS was investigated. As shown in Fig. 3, for H1 phosphorylation the enzyme was able to use all three nucleotide triphosphates, with apparent K₅₀ values of 75, 700, and 250 µM, respectively. Because the amount of p34αcyclin in these assays was too low to be measured, we were unable to determine a reliable specific activity value. However, at saturating nucleotide concentrations, similar amounts of the reaction were carried out as described under “Experimental Procedures” except that [γ-32P]ATP (314 dpm/pmol) was present at concentrations from 15 to 1000 µM. Products of the reaction were analyzed by polyacrylamide gel electrophoresis, and radiolabel incorporated into H1 was determined by liquid scintillation spectrometry. D, kinase reactions were performed as described under “Experimental Procedures” except that the concentration of [γ-32P]ATP (3 cpm/pmol) was 500 µM and H1 histone was present in concentrations from 0.0125 to 0.5 mg/ml.

**Fig. 3.** Kinetic parameters of MPF kinase for ATP, GTP, ATPγS, and H1 histone. A, kinase reactions were performed as described under “Experimental Procedures” except that [γ-32P]ATP was present in concentrations from 10 to 150 µM. B, kinase reactions were carried out as described under “Experimental Procedures” except that [γ-32P]GTP (2 cpm/pmol) was present at concentrations from 50 to 500 µM, and the products of the reaction were analyzed by polyacrylamide gel electrophoresis. C, kinase reactions were done as described under “Experimental Procedures” except that [γ-32P]ATP (314 dpm/pmol) was present at concentrations from 15 to 1000 µM. Products of the reaction were analyzed by polyacrylamide gel electrophoresis, and radiolabel incorporated into H1 was determined by liquid scintillation spectrometry. D, kinase reactions were performed as described under “Experimental Procedures” except that the concentration of [γ-32P]ATP (3 cpm/pmol) was 500 µM and H1 histone was present in concentrations from 0.0125 to 0.5 mg/ml.
**FIG. 4. Effect of divalent cations on MPF kinase activity.**

_A_., reactions were performed as described under "Experimental Procedures" except that the indicated concentrations of MgCl$_2$ were present. An additional 0.2 mM MgCl$_2$ was present in each reaction because of the addition of enzyme. _B_, reactions were carried out as described under "Experimental Procedures" except that no MgCl$_2$ was added and the indicated concentrations of MnCl$_2$ were present. The concentration of MgCl$_2$ and EGTA present due to the addition of enzyme was 0.3 and 0.1 mM, respectively. The incorporation of radioactivity into H1 at 0.2 mM MnCl$_2$ was 90% of that achieved in the same experiment in the presence of 15 mM MgCl$_2$. _C_, reactions were done as described under "Experimental Procedures" except that the concentration of MnCl$_2$ was 15 mM and the indicated concentrations of ZnCl$_2$ were present. The concentration of EGTA present due to the addition of enzyme was 25 μM.

**FIG. 5. Effect of pH and temperature on MPF kinase activity.**

_A_, kinase reactions were performed as described under "Experimental Procedures" except that the buffering agents (50 mM concentration) were as follows: MES at pH 6.0 and 6.5, Hapes at pH 7.0 to 8.5, and Bicine at pH 9.0 to 10.0. _B_, samples of MPF were incubated at the indicated temperatures for 5 min and then cooled to 0 °C. Then samples from each tube were assayed for H1 kinase activity as described under "Experimental Procedures." Results are expressed as percent of incorporation by the sample kept at 0 °C during the initial incubation.
MPF Kinase Characterization

![Graphs showing the effect of phosphatase inhibitors on MPF kinase activity.](image)

**TABLE I**

| Substrate            | MPF kinase | Growth-associated H1 kinase |
|----------------------|------------|-----------------------------|
| H1 histone           | 100        | 100                         |
| MAP-2                | 40         | 40                          |
| α-Casein             | 17         | 8                           |
| Inhibitor 1          | 4          | 6                           |

Kinase reactions were done as described under “Experimental Procedures” in the presence of H1 histone (0.5 mg/ml), MAP-2 (0.1 mg/ml), α-casein (1 mg/ml), or inhibitor 1 (6 μg/ml). Products of the reaction were analyzed by polyacrylamide gel electrophoresis. Results are expressed as percent incorporation of 32P into H1 histone.

No effect was seen with Ca²⁺/calmodulin (200 μM/20 nM) or with EGTA (1 mM) (data not shown). With Mg²⁺ as the metal ion, the pH optimum for activity was found to be 7.5 to 8.5 (Fig. 5A). In addition, as illustrated in Fig. 5B, the H1 kinase activity of MPF is very thermolabile. Incubation of the enzyme at temperatures above 30 °C caused a substantial decrease in activity, with incubation at 50 °C for 5 min resulting in a complete loss of activity.

MPF activity is highly unstable *in vitro* and often cannot be extracted from cells in the absence of β-glycerophosphate and sodium fluoride, compounds that are thought to act by inhibiting protein phosphatase activity (38). In fact, MPF is unable to cause nuclear envelope breakdown and chromosome condensation in oocytes or in the cell-free system in the absence of β-glycerophosphate. Although β-glycerophosphate is also known to have a marked stabilizing effect on phosphorylase b kinase (40), other work in this laboratory has shown that both β-glycerophosphate and NaF are potent inhibitors of ribosomal protein S6 kinase II from *Xenopus* eggs (32). To investigate the effect of these substances on p34<sup>cdc2</sup>, assays using H1 as substrate were carried out in the presence of varying concentrations of these compounds. As shown in Fig. 6, A and B, both β-glycerophosphate and NaF inhibited MPF kinase activity, with apparent IC₅₀ values of 75 and 7 mM, respectively. However, p-nitrophenyl phosphate, another widely used protein phosphatase inhibitor, had a slight stimulatory effect on MPF kinase activity (Fig. 6C). The fact that β-glycerophosphate is required for mitotic effects of MPF in the cell-free system yet inhibits the kinase activity *in vitro* suggests that the positive effect of β-glycerophosphate is directed at other components in the cell-free system. It also indicates the necessity of using different buffer systems to assay MPF as protein kinase activity *versus* nuclear breakdown.

As indicated in the introduction, both MPF and growth-associated H1 kinases contain homologs of p34<sup>cdc2</sup> as one component of their structure. This suggests that the substrate specificity and relative rates of phosphorylation of various substrates should be similar for both enzymes. To evaluate this prediction, the relative rates of phosphorylation of H1 histone, α-casein, inhibitor 1, and MAP-2 were compared for highly purified MPF and partially purified growth-associated H1 kinase. As shown in Table I, the relative activity of the two enzymes was similar against these substrates, with MAP-2 being phosphorylated at 40% of the rate of H1 histone, and the other substrates at levels below 20% of that with H1. Similar results were obtained when GTP was used as the phosphate donor (data not shown).

These results provide an initial characterization of the protein kinase component of MPF, p34<sup>cdc2</sup>. This kinase is unusual in its highly restricted substrate specificity and in its ability to use both ATP and GTP and also ATPγS. Other than growth-associated H1 kinase, which contains a p34<sup>cdc2</sup> homolog, only one other serine/threonine kinase, cassetin kinase II, has been found previously to utilize both ATP and GTP (41). However, that enzyme is markedly inhibited by heparin (41), whereas heparin did not inhibit MPF H1 kinase activity at concentrations up to 200 μg/ml (data not shown). The ability of the enzyme to use GTP may facilitate identification of substrates other than H1, since assays with GTP...
should significantly reduce the level of background phosphorylation by other kinases in partially purified preparations.

Studies with human cells have indicated that only about 5% of p34\(^{cdk2}\) is present as active protein kinase at mitosis (42, 43). This suggests that p34\(^{cdk2}\) is likely to have other functions besides control of M-phase, and indeed, p34\(^{cdk2}\) is known to have an obligatory role in the G1 phase of the yeast cell cycle (1). These different roles of p34\(^{cdk2}\) suggest that the kinase may associate with different components in different cells or in different phases of the cell cycle. Some evidence for this already exists since different proteins are co-purified or co-immunoprecipitated with p34\(^{cdk2}\) in budding yeast, fission yeast, Xenopus MPF, starfish egg MPF, HeLa cells, and growth-associated H1 kinase (20, 21, 23, 42, 43). At present the function of these associated components is unknown. In some cases these associated components seem to be substrates for p34\(^{cdk2}\), whereas in other cases they do not. Whether they affect the activity of p34\(^{cdk2}\) or its substrate specificity will require analysis of other purified preparations of p34\(^{cdk2}\) complexed with other proteins.

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