Identification of an Activator Required for Elevation of Maturation-promoting Factor (MPF) Activity by γ-S-ATP

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Abstract. Maturation-promoting factor (MPF) is a cell cycle control element able to cause cells to enter M-phase upon microinjection and will induce metaphase in nuclei incubated in cell extracts. Previous work has shown that MPF is composed of a complex between p34cdc2 protein kinase and a B-type cyclin. In the present work γ-S-ATP was found to cause activation of MPF activity in partially purified preparations, but this activation was lost upon chromatography on Matrex Green gel A. Readdition of other Matrex Green fractions to purified MPF restored the ability of γ-S-ATP to activate MPF for nuclear breakdown as well as phosphorylation of histone H1. Use of the system described here will facilitate study of p34cdc2 kinase activation and identification of elements involved in MPF regulation.

1. Abbreviations used in this paper: MG, Matrex Green; MPF, maturation-promoting factor; NEBD, nuclear envelope breakdown.

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Beach (1988) showed that in late G2 phase a minor fraction of p34<sup>cdc 2</sup> entered a high molecular mass complex with a 62-kD protein and the 13-kD product of the suc I gene. Formation of this complex was associated with elevated protein kinase activity, but it is not known if this complex represents MPF in HeLa cells. In the budding yeast Saccharomyces cerevisiae, a small fraction of the CDC 28 gene product, a functional homologue of cdc 2<sup>+</sup>, enters a high molecular mass complex with a 40-kD substrate and exhibits elevated protein kinase activity (Wittenberg and Reed, 1988). It is not known if this complex represents MPF in S. cerevisiae. An M-phase-activated H1 kinase has also been identified in several cell types as a complex of p34<sup>cdc 2</sup> with other proteins (Arion et al., 1988; Labbé et al., 1989b; Langan et al., 1989).

In addition to association with other components, phosphorylation of p34<sup>cdc 2</sup> has been identified as a regulatory mechanism controlling its protein kinase activity. Gautier et al. (1989) showed that in interphase Xenopus oocytes and activated eggs, p34<sup>cdc 2</sup> was highly phosphorylated, and its histone H1 kinase activity was low, whereas upon entry into M-phase in either meiosis or mitosis, p34<sup>cdc 2</sup> was dephosphorylated and its histone HI protein kinase activity became activated. Dorée and co-workers also reported apparent dephosphorylation of starfish oocyte p34<sup>cdc 2</sup> upon entry into M-phase (Labbé et al., 1989b). The phosphorylation occurs on both tyrosine and threonine residues, although quantitative removal of tyrosine phosphate does not activate mouse p34<sup>cdc 2</sup> (Dunphy and Newport, 1989; Morla et al., 1989). However, Gould and Nurse (1989) identified the phosphotyrosine site in S. pombe cdc 2 and showed mutation in that site caused mitotic catastrophe. In both frog and starfish, the kinetics of HI kinase activity changes are very similar to changes in MPF activity monitored by injection of oocytes. During these changes in protein kinase activity of MPF, no important change occurs in the amount of p34<sup>cdc 2</sup> present in the cell (Gautier et al., 1989; Labbé et al., 1989b). This suggests that the disappearance of MPF kinase activity during interphase represents inactivation of the kinase component, not its destruction.

Consistent with this concept, Dunphy and Newport (1988) found that MPF activity could be recovered from ammonium sulfate fractions of interphase egg extracts in the presence of adenosine (3'-O-thio)-triphosphate (γ-S-ATP) and a small amount of crude active MPF. In this paper we have investigated the activation of MPF by γ-S-ATP at different stages of purification and identified a protein activator required for activation by γ-S-ATP.

**Materials and Methods**

**Materials**

γ-S-ATP and histone HI were from Boehringer Mannheim Diagnostics, Inc., Houston, TX. DEAE-Sepharose and heparin-Sepharose were from Pharmacia Inc., Piscataway, NJ, and Matrex Green gel A was from Amicon Corp., Danvers, MA.

**Preparation of Metaphase MPF and Latent MPF**

Metaphase MPF was prepared as described by Lohka et al. (1988) except that the heparin-Sepharose column was eluted with 600 mM NaCl instead of 400 mM NaCl when γ-S-ATP was omitted from the preparation as indicated in the figure legends. A unit of MPF is defined as that amount in a volume of 50 μl that causes at least 20% of newly assembled pronuclei to undergo nuclear envelope breakdown (NEBD) within 2 h in a final volume of 75 μl containing 2 x 10<sup>5</sup> sperm nuclei/ml (Lohka et al., 1988). Latent MPF was prepared as described by Dunphy and Newport (1988) except that eggs were activated by treatment with the calcium ionophore A23187 (5 μg/ml in 50% medium OR2 for 10 min), followed by washing, and incubation in 10% OR2 until 30 min, at which point eggs were resuspended in centrifugation at 10,000 x g for 15 min. Pronuclear extracts for assaying MPF were prepared as described by Lohka and Maller (1985).

**Preparation of Activator Fraction**

The activator fraction was eluted in the 600-1,200 mM NaCl fraction from the Matrex Green gel A column used in MPF purification. Proteins in the pooled fractions derived from ~100 ml of eggs were precipitated by addition of an equal volume of 38 M ammonium sulfate, collected by centrifugation, dissolved in 0.5 ml of dialysis buffer, and dialyzed overnight. Dialysis buffer was 100 mM β-glycerophosphate, 5 mM EGTA, 15 mM MgCl<sub>2</sub>, 1 mM DTT, 20 mM Hepes, pH 7.5. The activator was stored at -70°C in small aliquots.

**Activation of Latent and Metaphase MPF**

60 μl of latent MPF was mixed with 3 μl of active, metaphase MPF containing 1 unit of activity and 3 μl of 6.6 mM γ-S-ATP. After incubation for 2 h at 22°C, MPF activity was assayed as described by Lohka et al. (1988). Controls were carried out with γ-S-ATP with latent or metaphase MPF alone or latent and metaphase MPF mixed without any γ-S-ATP. Activation of metaphase MPF was performed by incubating 18 μl of Matrex Green-purified MPF containing 1 U of activity with 45 μl of the activator fraction and 3 μl of 6.6 mM γ-S-ATP for 90 min at 22°C. Controls were carried out by incubation of MPF and activator without γ-S-ATP. MPF activity was then assessed under standard conditions as described above. In some experiments activation mixtures also contained 1 x 10<sup>6</sup> cpm of γ-<sup>32</sup>P-ATP and the products of the reaction were analyzed by SDS gel electrophoresis and autoradiography. HI histone kinase assays were carried out as described previously (Gautier et al., 1988).

![Figure 1. Activation of metaphase MPF or latent MPF by γ-S-ATP.](image-url)

Latent MPF was isolated as described in Materials and Methods from unfertilized eggs activated for 30 min by treatment with the calcium ionophore A23187. Active metaphase MPF was purified from nonactivated eggs through ammonium sulfate precipitation by the method of Lohka et al. (1988) and diluted to 0.7 U per addition so that it alone had no ability to cause NEBD in the absence of γ-S-ATP. Units of MPF were depicted as zero in the graph. After the indicated components were incubated in a final volume of 25 μl at 22°C for 120 min, various dilutions were added to 12.5 μl of cell-free extracts containing newly assembled nuclei and the extent of nuclear breakdown assessed by phase contrast and fluorescence microscopy. A unit of MPF activity was defined as the reciprocal of the highest dilution of MPF that caused at least 20% of nuclei to undergo NEBD. The data show crude active MPF can be further activated twofold by γ-S-ATP alone and latent MPF activation requires both active MPF and γ-S-ATP.
Results

In initial investigations, we carried out experiments similar to those described by Dunphy and Newport (1988). Ammonium sulfate fractions were prepared from unfertilized eggs in interphase after activation by treatment with the calcium ionophore A23187 (latent MPF). These fractions were then incubated with γ-S-ATP and a small amount of an ammonium sulfate fraction of MPF from metaphase-arrested unfertilized eggs (active metaphase MPF but an amount insufficient to cause any NEBD by itself in vitro). After 60 min, sperm pronuclei assembled in the cell-free system were added and NEBD was assessed after an additional 60 min by fluorescence microscopy. As reported originally by Dunphy and Newport (1988), we observed elevated MPF activity in the cell-free system when these three components were incubated together (Fig. 1). However, we carried out an additional control experiment not reported by Dunphy and Newport (1988) and found that γ-S-ATP alone was able to activate ammonium-sulfate purified metaphase MPF itself (Fig. 1). This indicated that the elevated MPF activity in the system reported by Dunphy and Newport (1988) was a mixture of effects of γ-S-ATP on the active metaphase MPF component itself as well as on the latent MPF component from interphase eggs. Therefore, subsequent experiments focussed on the activation of metaphase MPF from M-phase eggs. The dose dependence of γ-S-ATP-dependent activation of metaphase MPF was compared in the presence and absence of latent MPF as shown in Fig. 2. Consistent with Fig. 1, substantial activation of metaphase MPF by γ-S-ATP alone was evident at different metaphase MPF concentrations, but maximal activation of metaphase MPF by γ-S-ATP occurred at higher MPF concentrations than were required for maximal activation of latent MPF (Fig. 2). The extent of activation of metaphase MPF by γ-S-ATP alone varied considerably from one preparation to another, ranging from 1.5- to 4-fold with an initial MPF activity of 1 U/50 μl.

The metaphase MPF used by Dunphy and Newport (1988) and also in Figs. 1 and 2 is a crude ammonium sulfate preparation that is <10-fold purified over cytosol. In an attempt to analyze the system more rigorously, we have examined the activation of metaphase MPF itself in fractions from various steps of the MPF purification procedure that we have reported previously (Lohka et al., 1988). As shown in Fig. 3, metaphase MPF purified through TSK3,000SW chromatography was no longer able to be activated by γ-S-ATP alone, although it would still support activation of latent MPF from interphase eggs in the presence of γ-S-ATP. Subsequent experiments demonstrated the activating component was removed at the Matrex Green chromatography step. Whether the latent MPF preparation also contains the activating component removed from metaphase MPF by Matrex Green chromatography will require purification of the latent form of MPF.

The active metaphase MPF used in Figs. 1-3 was prepared according to the procedure of Lohka et al. (1988) that uses addition of γ-S-ATP to crude extracts and after ammonium sulfate precipitation. The γ-S-ATP-dependent activation of metaphase MPF in Figs. 1-3 was thus superimposed on a preparation that had already been incubated with γ-S-ATP. To maximize the effect of γ-S-ATP, we investigated the activation of metaphase MPF in ammonium sulfate preparations made without addition of γ-S-ATP. Although the recovery of metaphase MPF in such preparations was only 50% of that seen when γ-S-ATP was included, addition of γ-S-ATP caused a greater increase in MPF activity than when a γ-S-ATP incubation had occurred earlier in the purification (data not shown). Therefore, subsequent experiments used MPF preparations that had not been previously treated with γ-S-ATP. The time course of activation of such a metaphase MPF preparation and its dependence on γ-S-ATP concentration were analyzed, as shown in Fig. 4. The activating effect was maximal after 15 min of incubation and 3-150 μM γ-S-ATP was required for maximal activation.

Because the component required for activation of MPF was separated from MPF by Matrex Green (MG) gel chromatography (Fig. 3), we assessed the ability of fractions from the MG column to reconstitute the ability of metaphase MG MPF to be activated further by γ-S-ATP. As shown in

Figure 2. Dose-dependence for metaphase MPF activation of latent MPF. Different amounts of metaphase MPF were used in the activation of latent MPF as described in Fig. 1. The data show 0.8 U of metaphase MPF in combination with γ-S-ATP is sufficient to cause maximal activation of latent MPF. (●) γ-S-ATP plus latent MPF; (○) γ-S-ATP alone.

Figure 3. Removal of an activating component during MPF purification. Metaphase MPF was purified through TSK3,000SW chromatography, and analyzed for ability to activate latent MPF. (●) γ-S-ATP plus latent MPF; (○) ATP-γ-S alone. The data show the more highly purified MPF is not activated by γ-S-ATP alone (compare with Fig. 2) but can still activate latent MPF in the presence of γ-S-ATP.
and absence of metaphase MPF. A thiophosphorylated band of $M_r = 45$ kD is evident in both the metaphase MPF alone and the activator fraction alone, but the number of thiophosphorylated proteins visible when the twofractions are mixed is too great to permit identification of specifically enhanced thiophosphoproteins (not shown). Purified MPF contains a subunit of $M_r = 45$ kD (Lohka et al., 1988) which has been identified as a B-type cyclin and which can be thiophosphorylated by $\gamma$-S-ATP (Gautier et al., 1990). Preliminary experiments indicate that antibody to either cyclin B1 or B2, both of which are present in Mono S purified MPF (Gautier et al., 1990), can immunoprecipitate a 45-kD thiophosphorylated band in these extracts (Yamashita, S., and J. Gautier, unpublished results). However, under conditions where MPF is activated, the amount of radiolabeled thiophosphate in the 45-kD protein is unchanged.

These results demonstrate the presence of an activator of MPF in the cytoplasm of metaphase-arrested eggs. This activator is necessary for activation of partially purified MPF from metaphase-arrested eggs. The reaction is unlikely to represent an autoactivation because no MPF activity was evident in the activator fraction in the presence or absence of $\gamma$-S-ATP, and increasing the amount of MG-purified MPF in the system could not substitute for the activator fraction (data not shown). Dunphy and Newport (1988) reported and we have confirmed here that interphase eggs contain a latent form of MPF that conceivably could also be present in the activator fraction. To address this possibility, MPF activated by $\gamma$-S-ATP and the activator was diluted to give the same MPF concentration as initially (1 U/50 $\mu$L), and then fresh activator plus $\gamma$-S-ATP was added. No further increase in MPF activity was evident (data not shown), eliminating the possibility that latent MPF was present in the activator fraction.

Discussion

Two general approaches to study of the cell cycle have been productive to date. One approach has used the power of mo-

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**Figure 4.** Dependence of metaphase MPF activation on time and $\gamma$-S-ATP. (A) $\gamma$-S-ATP (300 $\mu$M) was mixed with metaphase MPF (0.8 U) prepared without $\gamma$-S-ATP for the indicated times at 22°C and then MPF activity was assayed in the cell-free system. The data show $\gamma$-S-ATP-dependent activation is maximal by 15 min. (B) $\gamma$-S-ATP concentration dependence. Metaphase MPF (0.8 U) was incubated with varying concentrations of $\gamma$-S-ATP at 22°C for 2 h and then assayed as described in Materials and Methods. The data show 150 $\mu$M $\gamma$-S-ATP is sufficient for maximal activation.

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**Figure 5.** Loss and restoration of MPF activation by $\gamma$-S-ATP. Aliquots of MPF fractions containing 1 U of activity from various stages of purification without initial $\gamma$-S-ATP treatment were incubated with $\gamma$-S-ATP, and assayed for activity as in Fig. 1. Purification from DEAE to MG revealed a loss of ability to be activated. However, readdition of the 0.6-1.2 M eluate from the MG column to MG-purified MPF restored the ability of $\gamma$-S-ATP to activate MPF (MG-MPF/activator). This material is referred to as the "activator" fraction. Incubation of the activator alone with $\gamma$-S-ATP did not produce any MPF activity.

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lecular genetics in both fission and budding yeast to identify genes critical for cell cycle control (see Lee and Nurse, 1988 for review). This work has identified p34<sup>cdc 2</sup> as a central regulator involved in the G<sub>S</sub> → M transition and uncovered a network of other cell cycle control genes that regulate cdc 2<sup>+</sup> activity, including the products of the wee 1<sup>+</sup>, nim 1<sup>+</sup>, suc 1<sup>+</sup>, cdc 25<sup>+</sup>, and cdc 13<sup>+</sup> genes (Russell and Nurse, 1987a, b; Solomon et al., 1988; Goebi and Byers, 1988; Hayles et al., 1986; Simanis and Nurse, 1985). A limitation to the genetic approach is that it is often difficult to obtain the functionally active protein products of the genes. This limitation is absent in the biochemical approach to the cell cycle, which has been used to purify MPF as a major cell cycle control element in the G<sub>S</sub> → M transition (Lohka et al., 1988). Both the genetic and biochemical approaches have converged on MPF in the light of evidence that MPF is a complex of p34<sup>cdc 2</sup> and p45<sup>cdc 28</sup>, homologues of two cell cycle control genes identified in yeast. In this paper we have continued the biochemical approach to cell cycle control, investigating in particular the mechanism of activation of MPF.

We have identified in metaphase MPF an “activator” component that is required for activation of MPF by γ-S-ATP.

Recent studies have shown that the activation of MPF by the activator fraction is also correlated with activation of the histones H1 kinase activity of cdc 2, as might be expected. In terms of kinase activation, p34<sup>cdc 2</sup> kinase activity in MPF has been found by several laboratories to be activated by dephosphorylation during the cell cycle both in vivo and in vitro (Gautier et al., 1989; Morla et al., 1989; Labbé et al., 1989c; Dunphy and Newport, 1989; Gould and Nurse, 1989). This biochemical finding is consistent with the genetic evidence in S. pombe that the function of the cdc 2<sup>+</sup> gene is inhibited by the product of the wee 1<sup>+</sup> gene, which is predicted to encode a serine/threonine protein kinase (Russell and Nurse, 1987a). The inhibitory function of this gene is itself inactivated by the product of another gene called nim 1<sup>+</sup>, which also is predicted to encode a serine/threonine protein kinase (Russell and Nurse, 1987b).

Given the high degree of conservation of mitotic controls it is therefore tempting to speculate that the requirement for γ-S-ATP for activation of MPF reflects the irreversible inactivation of wee 1<sup>+</sup> due to dephosphorylation by a homologue of the nim 1<sup>+</sup> gene. Alternatively, the activator could be a p34<sup>cdc 2</sup> phosphatase that was itself activated by dephosphorylation. Finally, the product of the cdc25<sup>+</sup> gene is involved in the timing of p34<sup>cdc 2</sup> activation and could potentially be involved here. These possibilities cannot be rigorously evaluated until the activator protein has been purified and characterized and the protein kinase that phosphorylates it identified.

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