Inactivation of p42 Mitogen-activated Protein Kinase Is Required for Exit from M-phase after Cyclin Destruction*

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By using cycling Xenopus egg extracts, we have previously found that if mitogen-activated protein kinase (p42 MAPK) is activated on entry into mitosis (M-phase), the extract is arrested with condensed chromosomes and spindle microtubules. Here we show that these arrested extracts have high levels of M-phase promoting factor (MPF, Cyclin B/Cdc2) activity, stabilized levels of Cyclin B, and sustained M-phase-specific phosphorylations. We also examined the role of p42 MAPK in DNA damage checkpoint-arrested extracts that were induced to enter M-phase by the addition of Cdc25C protein. In these extracts, Cdc25C protein triggers the abrupt, premature activation of MPF and entry into M-phase. MPF activity then drops suddenly due to Cyclin B proteolysis, just as p42 MAPK is activated. Unexpectedly, however, M-phase is sustained, as judged by maintenance of M-phase-specific phosphorylations and condensed chromosomes. To determine if this M-phase arrest depended on p42 MAPK activation, we added PD98059 (PD), an inhibitor of p42 MAPK activation, to egg extracts with exogenous Cdc25. Both untreated and PD-treated extracts entered M-phase simultaneously, with a sharp peak of MPF activity. However, only PD-treated extracts subsequently exited from M-phase and entered interphase. In PD-treated extracts, p42 MAPK was not activated, and the transition to interphase was accompanied by the formation of decondensed nuclei and the disappearance of M-phase-specific phosphorylations of proteins. These results show that although entry into M-phase requires the activation of MPF, exit from M-phase even after cyclin destruction, is dependent on the inactivation of p42 MAPK.

To date, a large body of research has led to the prevailing view that M-phase promoting factor (MPF), a complex of cyclin B/Cdc2) activation is required for entry into M-phase, and its inactivation is necessary for exit from M-phase (reviewed in Ref. 1). Studies have shown that p42 mitogen-activated protein kinase (p42 MAPK) activation maintains high levels of MPF activity and stabilizes Cyclin B (2–4), leading to the predominant belief that p42 MAPK must sustain M-phase solely by this mechanism.

We have previously shown that the activation of p42 MAPK in cycling Xenopus egg extracts by the addition of a constitutively active MAPK kinase (MEK) leads to an arrest of the cell cycle in either G2 or M-phase, depending on the timing of p42 MAPK activation (5). If p42 MAPK was activated in cycling egg extracts before entry into M-phase, the cell cycle was arrested in G2 (4–7). If p42 MAPK was activated on entry into M-phase, however, Cdc25C phosphatase was hyperphosphorylated (5, 7–9), and nuclear envelope breakdown (NEBD) and chromosome condensation (CC), all markers of M-phase, were sustained (5, 7).

Recently, we and others (7, 10) have shown that activation of p42 MAPK by Mos can lead to an M-phase arrest that is maintained even after the inactivation of MPF. We have further shown that MPF levels fall in these extracts due to mitotic cyclin proteolysis (7). Here, we show that the inactivation of p42 MAPK is required for exit from an M-phase arrest that is sustained after mitotic cyclin degradation and Cdc2 inactivation. In the process of initially characterizing recombinant Cdc25C proteins (wild type and a mutant that cannot be inhibited by 14-3-3 protein binding (11)‡), we have found that these proteins were able to drive interphase checkpoint-arrested egg extracts into an M-phase arrest as determined by cytology (NEBD and CC, see Refs. 11, 12, and this study) and maintenance of M-phase-specific phosphoproteins (this study).

Unexpectedly, although MPF was activated to high levels on entry into M-phase in these extracts, Cyclin B was rapidly proteolyzed leading to an abrupt fall in MPF levels, just as p42 MAPK activity appeared. The p42 MAPK activation was sustained throughout the M-phase arrest. To determine the role of p42 MAPK in this M-phase arrest, we added an inhibitor of MAPK activation to interphased extracts that were then driven into M-phase by the addition of Cdc25C protein. These extracts did not show p42 MAPK activation and did not arrest in M-phase. Instead, after a brief M-phase, the extracts entered interphase.

These results suggest that although the activation of MPF is required for entry into M-phase, p42 MAPK can sustain M-phase after MPF inactivation. Moreover, the inactivation of p42 MAPK, rather than MPF, is required for exit from M-phase.

EXPERIMENTAL PROCEDURES

Preparation of Egg Extracts and Sperm Nuclei—Cycling Xenopus egg extracts were prepared as described previously (5, 13). Briefly, dejellied Xenopus eggs were activated by electrical shock. At 21–23 min after activation, eggs were packed in extraction buffer (with protease inhibitors and cytochalasin B), over silicon oil, and extracts were made by

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1 The abbreviations used are: MPF, M-phase promoting factor; MAPK, mitogen-activated protein kinase; PD, PD98059; MEK, MAPK/extracellular signal-regulated kinase kinase; CC, chromosome condensation; NEBD, nuclear envelope breakdown; WT, wild type.

a A. S-S. Chau and E. K. Shibuya, unpublished observations.
centrifugation twice at 10,200 × g for 15 min at 4 °C. An ATP-regenerating system was added immediately, and supplemented extracts were kept on ice and used within 1 h.

Egg extracts for DNA damage checkpoint and Cdc25 protein addition experiments were prepared in the same way, except the extraction buffer was used at 4 °C. Sperm nuclei were prepared as described previously (14) and quantified prior to addition to extracts. For low sperm addition, preparations were diluted to 14,000 sperm nuclei/μl, and for high sperm additions, stocks of 49,000 nuclei/μl were prepared. Extracts of unfertilized eggs that are arrested at metaphase of meiosis were made as described previously (5) and used as M-phase controls.

Recombinant Protein Preparations—Constitutively active MEK protein (MEK(QP)) was expressed as an N-terminal glutathione S-transferase fusion protein and purified from Escherichia coli by glutathione-Sephrose affinity chromatography and then concentrated to 0.8–1.0 mg/ml, as described previously (5).

Cdc25 protein mutated at aspartic acid residue 287 (287 the serine required for 14-3-3 inhibition (11, 15, 16)) was generated via a two-step polymerase chain reaction procedure. A BamHI to ClaI fragment of Cdc25C with the Ser-287 to Ala substitution was generated using two oligonucleotides (ATGCGCAGAGGTCTGGACG and GGATAACGACAATCGATTATAG) to introduce an internal HindIII restriction site, and ATATAAAGCTTCTGGAACATGAGGTTGACGCC and GTAATAAGCTTCTGGAACATGAGGTTGACGCC to introduce the T to C polymerase chain reaction procedure. A N-terminal maltose-binding fusion protein in GTA and GATGAATATCTTGGGATCCCCCAT to introduce the T to C through the M. Reactions were incubated for at least 180 min of incubation, as judged by low levels of histone H1 kinase activity, interphase nuclei, and lack of MPM-2 reactive phosphoproteins (Fig. 2b). Hypophosphorylated Cdc25C(WT) protein could also rescue the checkpoint-arrested extracts in G2 by MEK(QP) (5).

We purified recombinant epitope-tagged Cdc25C proteins (wild-type (WT) and a mutant (S287A) in which serine at position 287 has been substituted with an alanine (11, 12)). When recombinant Cdc25C(S287A) protein was added to interphase-arrested extracts, the DNA damage checkpoint was overcome, with the activation of histone H1 kinase activity inducing entry into M-phase (Fig. 2c) (11, 12). We observed that the Cdc25C(WT) protein could also rescue the checkpoint-arrested extracts, but entry into M-phase occurred 20–30 min later than with Cdc25C(S287A) protein (data not shown), as has been previously reported (11). The S287A mutation abolishes the inhibition of Cdc25C by 14-3-3 protein binding (11, 15, 16, and data not shown), which explains why the addition of Cdc25C(S287A) to extracts induces M-phase earlier than the addition of Cdc25C(WT). However, the fact that Cdc25C(WT) addition to extracts is also able to induce M-phase suggests that the total level of Cdc25C (both endogenous and exogenous) exceeds the ability of the extract to inhibit all of the Cdc25C. Both Cdc25C proteins drive the interphase-arrested extracts into M-phase by removing the inhibitory tyrosine and threonine phosphorylations on Cdc2 (reviewed in Ref. 26).

The Cdc25C(S287A)-rescued extracts were arrested in M-phase, showing certain features that were similar to those observed in the MEK(QP)-induced M-phase-arrested extracts in Fig. 1. In both types of M-phase-arrested extracts, Cdc25C hyperphosphorylation was maintained (data not shown), MPM-2 reactive phosphoproteins persisted, and condensed chromosomes were associated with spindles. In contrast to the MEK(QP)-induced M-phase-arrested extracts, however, in the Cdc25C(S287A)-rescued extracts, histone H1 kinase activity dropped abruptly to interphase levels, and Cyclin B was proteolyzed (Fig. 2c). Interestingly, p42 MAPK was fully activated after the peak of histone H1 kinase activity in these Cdc25C(S287A)-rescued extracts (Fig. 2c, 90 min), and p42 MAPK activation was maintained throughout the M-phase arrest. Thus, in these extracts, maintenance of M-phase was correlated with sustained p42 MAPK activation, not high levels of the spindle assembly checkpoint and p42 MAPK activation are induced by high concentrations of nuclei and microtubule depolymerization (2, 23).

In addition to Cdc25C hyperphosphorylation (data not shown) (5), the extent of the other M-phase-specific phosphorylations in the arrested extract is shown by the sustained presence of MPM-2-reactive phosphoproteins, whereas these proteins are detected only transiently during M-phase in cycling extracts (Fig. 1c). The MPM-2 monoclonal antibody recognizes proteins when they undergo M-phase-specific phosphorylations (7, 24).

Cdc25C Drives Interphase Checkpoint-arrested Egg Extracts into an M-phase Arrest—It has been previously shown that egg extracts containing unreplicated or damaged DNA have an extended interphase and can be driven into M-phase by the addition of excess recombinant Cdc25C protein (11, 12). In the current study, we observed that egg extracts with unirradiated sperm nuclei added to 700 sperm/μl cycled slowly and did not enter M-phase until 140 min of incubation (Fig. 2a), a delay consistent with previous observations (11, 12, 25).

We then added UV-irradiated sperm nuclei (700 sperm/μl) to cycling egg extracts to trigger the DNA damage checkpoint in vitro (11, 12). These extracts were arrested in interphase for at least 180 min of incubation, as judged by low levels of histone H1 kinase activity, interphase nuclei, and lack of MPM-2-reactive phosphoproteins (Fig. 2b). Hypophosphorylated Cdc25C(WT) protein could also rescue the checkpoint-arrested extracts, but entry into M-phase occurred 20–30 min later than with Cdc25C(S287A) protein (data not shown), as has been previously reported (11). The S287A mutation abolishes the inhibition of Cdc25C by 14-3-3 protein binding (11, 15, 16, and data not shown), which explains why the addition of Cdc25C(S287A) to extracts induces M-phase earlier than the addition of Cdc25C(WT). However, the fact that Cdc25C(WT) addition to extracts is also able to induce M-phase suggests that the total level of Cdc25C (both endogenous and exogenous) exceeds the ability of the extract to inhibit all of the Cdc25C. Both Cdc25C proteins drive the interphase-arrested extracts into M-phase by removing the inhibitory tyrosine and threonine phosphorylations on Cdc2 (reviewed in Ref. 26).

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p42 MAPK Activation on Entry into M-phase Arrests the Cell Cycle with Stabilized MFP and Sustained M-phase-specific Phosphorylations—In Fig. 1a, we show that when p42 MAPK is activated by the addition of a constitutively active MEK (MEK(QP)) (5, 22) to cycling Xenopus egg extracts on entry into M-phase, the extract is arrested in M-phase with sustained histone H1 kinase activity, and Cyclin B2 is stabilized in the hyperphosphorylated form. Nuclear envelope breakdown (NEBD) and chromosome condensation (CC), markers of M-phase were maintained, with the condensed chromosomes associated with spindles (Fig. 1b) (5). These results are similar to
of MPF activity or stabilized Cyclin B.

p42 MAPK Inactivation Is Required for Exit from M-phase Arrest—To determine if activation of the p42 MAPK pathway was responsible for the M-phase arrest in egg extracts that had undergone cyclin destruction, the MEK-specific inhibitor, PD98059 (PD (5, 17)), or vehicle, Me2SO, was added to extracts containing 700 sperm/ml, and then Cdc25C(S287A) was added. As we observed with DNA damage checkpoint-arrested extracts, Cdc25C(S287A) and Me2SO addition to egg extracts caused an accelerated entry into M-phase (data not shown) and subsequent arrest. In this reaction, the M-phase arrest was accompanied by maintenance of p42 MAPK activation, MPM-2-reactive phosphoproteins (Fig. 3a), hyperphosphorylated Cdc25C (both endogenous and exogenous, not shown), and NEBD and CC (Fig. 3c). Similar to the DNA damage checkpoint-arrested extracts rescued by Cdc25C(S287A) (Fig. 3c), histone H1 kinase activity levels peaked on entry into M-phase, abruptly dropped to interphase levels, and then started to increase slowly (Fig. 3a). Again, p42 MAPK was fully activated just after the peak of histone H1 kinase activity. The fall in histone H1 kinase activity coincided precisely with proteolysis of Cyclin B2, and then Cyclin B2 began to accumulate as levels of histone H1 kinase activity rose (Fig. 3a).

When PD was added to egg extracts with 700 sperm/ml and Cdc25C(S287A), entry into M-phase occurred at the same time as in extracts without PD, with a peak of histone H1 kinase activity followed by a sudden drop to interphase levels (Fig. 3, a and b). In these PD-treated extracts, Cyclin B2 was proteolyzed, and then Cyclin B2 accumulated later than in extracts without PD (Fig. 3b). In contrast, however, p42 MAPK activation was completely inhibited by PD in these extracts (5), and after entering M-phase (as determined by the appearance of MPM-2-reactive phosphoproteins, and NEBD and CC (Fig. 3e)), the extracts exited M-phase and entered interphase. Transition to interphase was accompanied by decondensing nuclei (Fig. 3c), disappearance of M-phase-specific phosphoproteins (Fig. 3b), and dephosphorylation of Cdc25C (both endogenous and exogenous, not shown).

Taken together, our results show that although the activation of Cyclin B-Cdc2 complexes is required for entry into M-phase, p42 MAPK inactivation is required for exit from M-phase and transition to interphase. Moreover, these results also suggest that although entry into M-phase is required for the phosphorylation of Cdc25C and other MPM-2-reactive phosphoproteins, the sustained phosphorylations observed in M-phase arrest are maintained by p42 MAPK activation.
FIG. 2. A mutant Cdc25C protein drives DNA damage checkpoint-arrested egg extracts into a sustained M-phase arrest, with constitutive activation of p42 MAPK. We expressed and purified from E. coli a mutant Cdc25C protein in which the serine at position 287 was mutated to an alanine. To test this construct, we induced the DNA damage checkpoint in egg extracts by UV-irradiating sperm nuclei prior to addition to reactions at 700 nuclei/μl. Untreated sperm + buffer (a), UV-irradiated sperm + buffer (b), or UV-irradiated sperm + Cdc25(S287A) (c) was added to reactions, and samples were taken starting at 60 min of incubation. Samples were immunoblotted with phosphotyrosine antibodies (upper panels), Cyclin B2 antibodies (second panels), and MPM-2 antibodies (third panels). Bold lines below MPM-2 blots indicate time points when NEBD, CC, and mitotic microtubules were observed. Graphs below blots show phosphorimage quantitation of histone H1 kinase assays.

DISCUSSION

The major findings of this study are that the activation of p42 MAPK maintains M-phase arrest after the inactivation of MPF by Cyclin B destruction and that the inactivation of p42 MAPK is necessary for exit from M-phase. Recently, we have shown that when p42 MAPK is constitutively activated in cycling Xenopus egg extracts after the peak of MPF kinase activity, there is brief drop in Cdc2 activity due to Cyclin B (7). These results and those of the present work are unexpected given that the M-phase arrest in egg extracts previously observed in response to activators of the p42 MAPK (see Refs. 4 and 5, and Fig. 1, this study) or induction of the spindle assembly checkpoint (2, 23) exhibited sustained H1 kinase activity with stabilized B-type cyclins (2, 4, 23).

There are similarities between the M-phase-arrested extracts as represented in Fig. 1, and the M-phase-arrested extracts in Figs. 2 and 3 of this study. In all of the extracts, MPF is activated on entry into M-phase, and M-phase-specific phosphoproteins appear and are sustained. In addition, nuclei undergo NEBD and CC and remain in this state. By these criteria alone, it would be reasonable to predict that maintenance of MPF activity was solely responsible for the M-phase arrest in all of these extracts. However, the data in Figs. 2 and 3 clearly demonstrate that M-phase can be sustained by p42 MAPK after Cyclin B proteolysis and the fall of MPF activity to interphase levels, suggesting that p42 MAPK has additional functions later in M-phase that can maintain cell cycle arrest.

Differences in the timing of p42 MAPK activation in these extracts may account for these apparent contradictory findings. When the activation of p42 MAPK by MEK(QP) occurs during entry into M-phase coincident with MPF activation, high levels of MPF activity are maintained, and Cyclin B is stabilized (Fig. 1). In contrast, when interphase extracts are driven into M-phase by Cdc2/S287A (Figs. 2 and 3), p42 MAPK is fully activated after the peak of histone H1 kinase activity, at a time when MPF activity is rapidly falling. Therefore, in extracts driven into M-phase by Cdc25C protein addition there appears to be a dissociation between the cycle of MPF activation and inactivation and the timing of p42 MAPK activation. Under these conditions, p42 MAPK clearly sustained M-phase even after cyclin destruction. Similarly, in all of our cycling extracts studied to date (5, 7), the transient activation of p42 MAPK always occurred after the peak of MPF activity, and nuclei in M-phase continued to be present (post-cyclin destruction) in all samples that contained active p42 MAPK. Also, the addition of PD to cycling extracts shortened the duration of M-phase (judged by cytology) as compared with that observed in untreated cycling extracts (data not shown, see Ref. 10). Together with the results presented here, these observations suggest that the brief transient of p42 MAPK activation in normally cycling extracts can also extend M-phase after the inactivation of MPF by cyclin destruction.

After proteolysis, Cyclin B2 reappeared sooner in untreated extracts than in PD-treated extracts, suggesting that the duration of cyclin destruction in extracts with activated p42 MAPK was shorter than in extracts with unactivated p42 MAPK. These results are consistent with the results of previous studies that p42 MAPK activation can prevent the initiation of cyclin destruction (Fig. 1, this study, and Refs. 2 and 3). Our results further suggest that p42 MAPK activation can also turn off the destruction pathway once it has been activated (Figs. 2c and 3).

We also observed that histone H1 kinase activity reappears sooner in untreated extracts than in PD-treated extracts (Fig. 3). One explanation for this increase in activity could be that Cdc25C (both endogenous and exogenous) is stabilized in an active state by p42 MAPK in untreated extracts. As a result, as newly synthesized B-type cyclins (mitotic cyclins are continuously synthesized throughout the cell cycle, see Ref. 27) form complexes with Cdc2, these complexes could be activated immediately due to the activated exogenous and endogenous Cdc25C. There are two p42 MAPK consensus sites in Cdc25C, and we are presently exploring the possible role of these sites in the stabilization of Cdc25C hyperphosphorylation and activity.

Recently, it has been shown that activated MAPKs (p42 and p44) have been detected at kinetochores and at the periphery of condensed chromosomes during M-phase in somatic mammalian cells (28, 29). These results suggest that active p42 MAPK in Xenopus egg extracts could be similarly localized to chromatin during M-phase in cycling Xenopus egg extracts and in the Cdc25-induced M-phase-arrested extracts reported here. However, the maintenance of MPM-2-reactive phosphoproteins in p42 MAPK-arrested extracts suggests that M-phase conditions are uniform throughout the extract and extend beyond a simple localized effect on chromatin.

Our results provide strong evidence that although the activation of MPF-Cyclin B-Cdc2 complexes is required for entry into M-phase, once established M-phase can be sustained by
p42 MAPK activation and does not require the maintenance of MPF activity. Therefore, whereas the activation of MPF drives the cell cycle from interphase into M-phase, it appears that the inactivation of p42 MAPK, in addition to inactivation of MPF, is required for exit from M-phase.

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**FIG. 3.** Exit from M-phase arrest induced by Cdc25C(S287A) is dependent on inactivation of p42 MAPK and not MPF activity. Sperm nuclei (700 nuclei/μl) were added to egg extracts with Cdc25C(S287A) protein either without (a) or with (b) the MEK inhibitor, PD98059 (PD). Samples were taken starting at 60 min of incubation. Samples were immunoblotted with phosphotyrosine antibodies (upper panels), Cyclin B2 antibodies (second panels), and MPM-2 antibodies (third panels). Bold lines below MPM-2 blots indicate time points when NEBD, CC, and mitotic microtubules were observed. Graphs below blots show phosphorimage quantitation of histone H1 kinase assays. c–f, fluorescence images (Hoechst); bar = 10 μm. c, decondensed nucleus in sample taken at 60 min from reaction in a (shaded arrowhead). d, condensed chromosomes in sample taken at 180 min from reaction in a (solid arrowhead). e, condensed chromosomes in sample taken at 90 min from reaction in b (solid arrowhead). f, decondensed nucleus in sample taken at 120 min from reaction in b (shaded arrowhead).
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