Mitotic Effects of a Constitutively Active Mutant of the *Xenopus* Polo-Like Kinase Plx1

YUE-WEI QIAN, ELEANOR ERIKSON, AND JAMES L. MALLER*

Howard Hughes Medical Institute and Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado 80262

Received 27 May 1999/Returned for modification 24 June 1999/Accepted 2 August 1999

During mitosis the *Xenopus* polo-like kinase 1 (Plx1) plays key roles in the activation of Cdc25C, in spindle assembly, and in cyclin B degradation. Previous work has shown that the activation of Plx1 requires phosphorylation on serine and threonine residues. In the present work, we demonstrate that replacement of Ser-128 or Thr-201 with a negatively charged aspartic acid residue (S128D or T201D) elevates Plx1 activity severalfold and that replacement of both Ser-128 and Thr-201 with Asp residues (S128D/T201D) increases Plx1 activity approximately 40-fold. Microinjection of mRNA encoding S128D/T201D Plx1 into *Xenopus* oocytes induced directly the activation of both Cdc25C and cyclin B-Cdc2. In egg extracts T201D Plx1 delayed the timing of deactivation of Cdc25C during exit from M phase and accelerated Cdc25C activation during entry into M phase. This supports the concept that Plx1 is a "trigger" kinase for the activation of Cdc25C during the G2/M transition. In addition, during anaphase T201D Plx1 reduced preferentially the degradation of cyclin B2 and delayed the reduction in Cdc2 histone H1 kinase activity. In early embryos S128D/T201D Plx1 resulted in arrest of cleavage and formation of multiple interphase nuclei. Consistent with these results, Plx1 was found to be localized on centrosomes at prophase, on spindles at metaphase, and at the midbody during cytokinesis. These results demonstrate that in *Xenopus laevis* activation of Plx1 is sufficient for the activation of Cdc25C at the initiation of mitosis and that inactivation of Plx1 is required for complete degradation of cyclin B2 after anaphase and completion of cytokinesis.

Progression through the eukaryotic cell cycle is controlled through the periodic activation or inactivation of various cyclin-dependent protein kinases (cdk's) at specific points in the cycle (26). The fidelity of events in a given cell cycle phase is monitored by checkpoints, which control a signaling system that can delay cell cycle progression and changes in cdk activity (5, 9). One of the best-understood checkpoints blocks activation of the cyclin B-Cdc2 complex in G2 phase if DNA replication is incomplete (see reference 27 for a review). This block to mitotic entry requires that the phosphatase Cdc25C remain inactive. Throughout late S and early G2 phases, cyclin B is kept catalytically inactive by phosphorylation of Thr-14 and Tyr-15 in the ATP-binding site. This phosphorylation and inactivation are catalyzed by the protein kinases Wee1 and Myt1 (22, 29), and dephosphorylation and activation of cyclin B-Cdc2 are catalyzed by the phosphatase Cdc25C (6, 14, 21). Activation of Cdc25C requires phosphorylation on specific serine and threonine sites, which fails to occur if DNA synthesis is incomplete and the replication checkpoint is activated (13, 16). These considerations have focused attention on the phosphorylation pathway by which Cdc25C becomes activated at the G2/M transition. Cyclin B-Cdc2 can phosphorylate and activate Cdc25C, forming a positive feedback loop that contributes to the abrupt transition from G2 into M phase (10, 12). However, a variety of evidence indicates that in *Xenopus* initial phosphorylation and activation of Cdc25C result from activation of the polo-like kinase (plk) Plx1. Plx1 can phosphorylate and activate Cdc25C in vitro (15), and in vivo activation of Plx1 is concurrent with the activation of Cdc25C (30). Moreover, inhibition of Plx1 delays the activation of Cdc25C, and an elevated level of Plx1 accelerates the rate of Cdc25C activation (30). Plx1 is also activated by phosphorylation, and the newly identified *Xenopus* polo-like kinase 1 (xPlkk1) is able to phosphorylate and activate Plx1 in vitro (31). The xPlkk1 protein itself is also activated by phosphorylation, and in vivo activation of xPlkk1 coincides with the activation of Plx1. Moreover, an elevated level of xPlkk1 accelerates the timing of activation of Plx1 and the transition from the G2 to the M phase of the cell cycle (31). Both Plx1 and xPlkk1 might be subject to inhibition when the DNA replication checkpoint is activated.

In addition to the role for plk's at the G2/M transition, in a variety of organisms, including *Xenopus*, plk's also have other roles in mitosis. One important role in yeast, *Drosophila*, *Xenopus*, and mammalian cells is the requirement of plk function for bipolar spindle formation (7, 8, 17, 28, 30, 36). In the absence of plk function, monopolar spindles form, and localization studies show that plk's can be found on centrosomes and kinetochores at metaphase (30, 35). plk's appear to continue to function in mitosis even after the metaphase/anaphase transition has been triggered. In *Xenopus* egg extracts, addition of a catalytically inactive (kinase-dead) form of Plx1 blocks the degradation of B-type cyclins, and the system remains in M phase with high histone H1 kinase activity (4). In budding yeast cells the plk homolog Cdc5p is normally degraded by the anaphase-promoting complex (APC) (3, 33), and in *Xenopus* Plx1 activity decreases late in mitosis (30). Overexpression of Cdc5p results in increased degradation of certain CIBs, suggesting that degradation of Cdc5p is required to turn off the degradation of B-type cyclins by the APC (3). In both *Saccharomyces cerevisiae* and *Drosophila*, plk's are localized to the midbody and plk function appears to be required for cytokinesis (2, 19, 20). Most of the information obtained to date about the function of plk's has come from studies with loss-of-function mutants or
inhibitory antibodies. To learn more about the function of plk's it was imperative to generate a constitutively active mutant of Plxl and determine its effects on mitosis, as reported here.

MATERIALS AND METHODS

Manipulation of oocytes and eggs. Xenopus laevis was obtained from Xenopus I (Ann Arbor, Mich.). Techniques for dissection and culture of oocytes, in vitro fertilization of eggs, culture of embryos, and microinjection, have been described elsewhere (30).

Mutagenesis. The SI2D, T140D, T201D, T205D, S227D, S128A, and T201V mutants of Plxl were created by PCR with pairs of oligonucleotides with the following sequences: GAGGAGGGACCTGTTGGAGCTCAAGAG and CCACAACCTCGTCTGCAGCACATACTGCGCTACAGAG and ATGTGCTGACCGACGCTGTCGCTCAGCACATC, CATAATGTTGGAGCTCAAGAG and CAGATCGTCGACCGACGCTGTCGCTCAGCACATC, CAAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG, GAGGAGGGACCTGTTGGAGCTCAAGAG and CCAACTG. The SI2D/T201D and S128A/T201V mutants were created by PCR with two pairs of the above oligonucleotides corresponding to S12D and T201D and to S128A and T201V, respectively.

Immunoprecipitation, immunoblotting, and kinase assays. Stage Vl oocytes injected with mRNA encoding Plxl or the various Plxl mutants, tagged at the COOH terminus with FLAG, were lyzed, the extracts were immunoprecipitated with anti-FLAG M2-agarose beads (Sigma), and Plxl activity was assayed by phosphorylation of α-casein (30). Characterization of antibodies generated by this laboratory against cyclins, Cdc25C, and Plxl and used for immunoblotting has been detailed (13, 30, 32). Anti-phospho-mitogen-activated protein (MAP) kinase (9101) was from New England Biolabs, and anti-Mos (C237) was from ImmunoResearch. Confocal microscopy was performed with an MRC-600 microscope (Bio-Rad).

RESULTS

In vitro kinase activity of Plxl mutants expressed in oocytes. The plk's are highly conserved among different species, and their activity can be regulated by transcription, biosynthesis, and degradation, intracellular localization, and phosphorylation (8, 18, 30, 36). During meiotic maturation (the G/M transition) in Xenopus oocytes Plxl is activated by phosphorylation on both serine and threonine residues (30). Because plk's from several different organisms are activated by phosphorylation, it is plausible that the phosphorylation sites essential for their activation are also conserved. When the amino-terminal catalytic domain of Plxl is compared with those of mammalian Plk, Drosophila polo, Saccharomyces cerevisiae Cdc5p, and Schizosaccharomyces pombe plo1, several conserved serine and threonine residues are evident (Fig. 1). In many protein kinases substitution of a phosphorylated residue with a negatively charged aspartic acid residue can mimic the effect of phosphorylation on enzyme activity (11, 23). Therefore, five of these conserved residues were individually mutated to an aspartic acid residue, and the effect on Plxl activity was assessed.

To analyze the activity of ectopically expressed Plxl and its mutants, stage VI oocytes were microinjected with the various FLAG-tagged Plxl mRNAs, and anti-FLAG immunoprecipitates were used for casein kinase assays. Immunoprecipitates prepared from stage VI (G2 phase) oocytes expressing wild-type (WT) FLAG-tagged Plxl had very low casein kinase activity, whereas immunoprecipitates prepared from the corresponding mature oocytes (M phase) had at least a 40-fold increase in kinase activity toward casein. No casein kinase activity was observed in immunoprecipitates prepared from either stage VI or mature oocytes injected with buffer (Fig. 2A). Casein kinase activity in the immunoprecipitates was linear with time and amount of extract (data not shown). These results indicate that the kinase assay with anti-FLAG immunoprecipitates is specific for the FLAG-tagged ectopically expressed Plxl protein.

Mutation of Ser-128 to Asp (S128D) or of Thr-201 to Asp (T201D) increased substantially the kinase activity of Plxl toward casein, whereas mutation of Thr-140, Thr-205, or Ser-227 to Asp (T140D, T205D, and S227D) did not alter Plxl activity
significantly (Fig. 2B). Constitutive activity after mutation of Thr-201 to Asp in Plx1 is consistent with a previous report that mammalian Plk with a Thr-Asp mutation at the equivalent site has elevated activity (19). This residue is in the activation loop between protein kinase subdomains VII and VIII, and phosphorylation within this loop results in the activation of several protein kinases including Cdc2 and Mek1 (11, 23, 25). To generate an even more active Plx1 mutant, both Ser-128 and Thr-201 were substituted with Asp (S128D/T201D), mRNA was injected, and Plx1 activity was assayed. The S128D/T201D double mutant displayed 40-fold increased casein kinase activity (Fig. 2B).

Thr-201 is required for activation of Plx1. The results above suggested that phosphorylation of both T201 and S128 might be important for activation of Plx1 in vivo. To assess this possibility, mRNAs encoding T201V Plx1 or S128A Plx1 were injected into resting oocytes. After 2 h of incubation, progesterone was added to trigger the G2/M transition. After nuclear (germinal vesicle) breakdown, recombinant Plx1 was recovered on anti-FLAG beads and kinase activity was assessed (Fig. 3A). The T201V mutant exhibited essentially no activation in response to progesterone, whereas the S128A mutant was activated substantially. We have recently purified and cloned a protein kinase, termed xPlkk1, that is able to phosphorylate and activate Plx1 in vitro and that accelerates the activation of Plx1 in vivo (31). Both T201 and S128 are preceded by three basic residues and followed by a hydrophobic residue (Fig. 1). Therefore, synthetic peptides encompassing these residues were synthesized, and their ability to serve as substrates for xPlkk1 in vitro was evaluated in comparison with myelin basic protein (MBP) (Fig. 3B). The T201 peptide was phosphorylated almost as well as MBP, whereas the S128 peptide was not significantly phosphorylated, even though the sequences surrounding these residues are highly conserved. These results suggest that phosphorylation of T201, but not S128, by xPlkk1 is required for activation of Plx1.
Previous results (30) have shown that endogenous Plx1 remains highly activated after GVBD throughout both meiosis I and II in Xenopus oocytes. However, whether meiotic events beyond GVBD are normal in the presence of constitutively active Plx1 remains to be determined.

Constitutively active Plx1 attenuates the degradation of cyclin B2. In S. cerevisiae, the activity of plk’s is required for activation of the APC, which degrades cyclins and other proteins to drive the metaphase/anaphase transition and exit from mitosis (3, 33). This function of plk is likely to be conserved, inasmuch as addition of kinase-dead Plx1 to Xenopus egg extracts blocks degradation of cyclins and exit from M phase (4). In S. cerevisiae, the plk homolog Cdc5p is itself degraded by the APC during exit from mitosis (3, 33). Accumulation of particular B-type cyclins is reduced when Cdc5p activity is increased, and this effect is blocked by certain mutations in the APC (3). To determine whether Plx1 also affects cyclin reaccumulation after exit from M phase, metaphase-arrested CSF extracts were supplemented with T201D Plx1 purified from baculovirus-infected Sf9 cells. After addition of Ca2+ to trigger the metaphase/anaphase transition and exit from M phase, the levels of the cyclins A1, B1, and B2, the histone H1 kinase activity, and the activity of Cdc25C, as judged by its electrophoretic mobility, were monitored (Fig. 5). The extract containing constitutively active Plx1 had approximately fivefold-higher Plx1 activity than the endogenous level, and this level of activity remained constant throughout the experiment (data not shown). There was no effect of activated Plx1 on the rate of reaccumulation of any of the cyclins. However, constitutively active Plx1 delayed slightly the inactivation of Cdc25C during exit from mitosis and caused a much earlier activation of Cdc25C during the next mitosis. These effects were mirrored by changes in histone H1 kinase activity of Cdc2 and support the idea that Plx1 functions as a “trigger” kinase for the activation of Cdc25C during entry into mitosis. T201D Plx1 had a slight effect on the degradation of cyclin A1 and B1 (Fig. 5); however, the degradation of cyclin B2 was significantly attenuated and a larger fraction of the protein was in a shifted, phosphorylated form. This effect of activated Plx1 was consistently seen in five independent experiments. In agreement with the report of Descombes and Nigg (4), kinase-dead Plx1 blocked degradation of all three cyclins.

Constitutively active Plx1 causes cleavage arrest. The activity of Plx1 increases dramatically at the G2/M transition, remains high during mitosis, and decreases during cytokinesis due to dephosphorylation (30). In S. cerevisiae, plk activity declines after exit from mitosis due to APC-mediated degradation (3, 33). In both yeast and Drosophila, plk’s play a role in cytokinesis (2, 7, 19, 28), and plk’s have been localized at the midbody in several organisms. If downregulation of the activity of Plx1 in telophase is essential for exit from mitosis, expression of constitutively active Plx1 might result in a defect in cytokinesis. To test this hypothesis, one blastomere of a two-cell embryo was injected with mRNA encoding either WT Plx1 or S128D/T201D Plx1. The uninjected blastomere served as an additional control. Expression of S128D/T201D Plx1 in the embryo caused cleavage arrest, resulting in large cells, whereas overexpression of WT Plx1 had no effect relative to uninjected controls (Fig. 6A). To examine the defects within the embryo, the embryo was sectioned and the DNA was stained with SYTOX Green. As shown in Fig. 6B, the large cells resulting from expression of S128D/T201D Plx1 contain multiple enlarged nuclei. This suggests, but does not prove, that cytokinesis is blocked when the activity of Plx1 remains high.

Localization of Plx1 is consistent with its multiple functions during mitosis. Based on previous studies and the data presented here, Plx1 plays multiple essential roles during mitosis. It activates Cdc25C, leading to cyclin B-Cdc2 activation and mitotic entry (Fig. 4), it is required for organization of bipolar

A

![Graph](http://mcb.asm.org/)

**FIG. 4.** Induction of oocyte maturation by S128D/T201D Plx1. Stage VI oocytes were microinjected with 40 ng of mRNA encoding either WT Plx1 or S128D/T201D Plx1, and groups of six oocytes were frozen at the indicated times. (A) Extracts were prepared, and histone H1 kinase activity was determined. Symbols: □, WT Plx1; ■, S128D/T201D Plx1. (B) Samples of extracts were subjected to immunoblotting with anti-Cdc25C, anti-phospho-MAP kinase, and anti-Mos antibodies as indicated. Upper panel of each pair, WT Plx1 (mRNA injected); lower panel of each pair, WT Plx1 (mRNA injected).
spindles (17, 30, 35), it is necessary for activation of the APC (3, 4, 33), and its persistent activation causes a cleavage arrest (Fig. 6). Entry into mitosis begins with breakdown of the nuclear envelope while anaphase is initiated from the metaphase plate and cytokinesis from the midbody. This suggests that the localization of Plx1 might change during different stages of mitosis. To analyze this directly, subcellular localization of Plx1 during mitosis was examined by confocal microscopy (Fig. 7).

Plx1 is localized to centrosomes early in mitosis (Fig. 7d to f), consistent with its role in Cdc25C activation and the organization of bipolar spindle assembly; just before anaphase it is concentrated on the metaphase plate (Fig. 7g to i), consistent with its role in activation of the APC; and late in mitosis it is localized to the midbody (Fig. 7Ap to r), consistent with a role in cytokinesis (Fig. 6).

**DISCUSSION**

Several lines of evidence support the hypothesis that Plx1 is a trigger kinase that initiates the positive feedback loop between Cdc25C and cyclin B-Cdc2. First, Plx1 is able to bind, phosphorylate, and activate Cdc25C in vitro (reference 15 and our unpublished data). Second, Plx1 is activated with the same kinetics as Cdc25C during oocyte maturation (30). Third, inhibition of Plx1 in vivo with antibodies or by overexpression of kinase-dead Plx1 significantly delays the activation of Cdc25C, and immunodepletion or neutralization of Plx1 with antibodies suppresses the activation of Cdc25C (1, 30). Fourth, Cdc25C itself can overcome the inhibitory effect of Plx1 antibody (30). Finally, as shown here, in resting oocytes constitutively active Plx1 is sufficient to activate Cdc25C and initiate the G2/M transition, and in egg extracts it markedly accelerates Cdc25C activation and entry into M phase.

The results presented here indicate that both Ser-128 and Thr-201 residues play important roles for Plx1 activity. Mutation of Thr-201 to valine (T201V) abolished activation of Plx1, whereas mutation to aspartic acid conferred significant constitutive activity. Moreover, a synthetic peptide encompassing Thr-201 was a good substrate for Plk1 in vitro, and Plx1 activated in vivo contains phosphothreonine. T201V is in the activation loop, and phosphorylation of the corresponding residue is associated with activation of many other kinases. These considerations suggest that T201 phosphorylation in vivo is required for Plx1 activation. In contrast, the Ser-128 site was not phosphorylated as a synthetic peptide in vitro nor did a mutant at this site (S128A) suffer any impairment in activation...
Nevertheless, the S128D mutant did display significant constitutive activity and contributed greatly to the high activity of the S128D/T201D double mutant. An understanding of the role of Ser-128 on the activity of Plx1 will require knowledge of the three-dimensional structure of the enzyme.

The work presented here with kinase-dead enzyme confirms the requirement of Plx1 for activation of the APC and exit from mitosis (4). Moreover, new data shows that high T201D Plx1 activity during exit from mitosis and progression into the subsequent mitosis did not affect the reaccumulation of mitotic cyclins, suggesting that, unlike the situation in S. cerevisiae, inactivation of Plx1 is not required for inactivation of the APC in G1. However, the degradation of cyclin B2 was attenuated by constitutively active Plx1 whereas the degradation of cyclin B1 and A1 was much less affected. Previous work has suggested that cyclin B2 degradation in X. laevis is regulated differently from that of cyclin B1 in terms of a requirement for binding to Cdc2 (34), and in S. cerevisiae overexpression of plk affects the degradation of some Clbs but not others (3, 33). Our results suggest that in Xenopus Plx1 regulates degradation of cyclin B2 differently from that of cyclin B1.

In addition to a role for plk during entry into mitosis, a variety of evidence points to an essential role for plk’s in cytokinesis. Disruption of the fission yeast polo-like kinase gene, plo1, leads not only to a failure of spindle formation but also to a failure to form either an actin ring or a septum (28). Moreover, overexpression of either plo1 in S. pombe or Plk in S. cerevisiae induces ectopic septal structures (19, 28). Mutations in the Drosophila polo gene also cause defects in the early events of cytokinesis at various stages of spermatogenesis (2).

FIG. 6. Overexpression of constitutively active Plx1 causes cleavage arrest in early embryos. (A) Cleavage arrest in an injected blastomere. mRNA encoding either WT Plx1 (right) or S128D/T201D Plx1 (left) was microinjected into one blastomere of a two-cell embryo, and embryonic development was monitored with a dissecting microscope. (B) An arrested embryo as shown in panel A (left) was fixed, sectioned, and stained with SYTOX Green as described in Materials and Methods. Bar, 100 μm.
These results indicate that activity of plk’s is essential for the initiation of cytokinesis. Interestingly, in the current studies, expression of constitutively active Plx1 in Xenopus embryos led to a cleavage arrest that could result from defects in cytokinesis. It is likely that the defects observed here occur in the late events of cytokinesis. Consistent with this idea, in Xenopus embryos Plx1 is activated for entry into mitosis, kept high during mitosis, and deactivated after exit from mitosis during completion of cytokinesis (30). Deactivation of Plx1 may be essential for complete exit from mitosis or for completion of cytokinesis. Together, these results suggest that the initiation of cytokinesis requires the activity of plk’s, and the execution or completion of cytokinesis requires their inactivation. Although cleavage arrest is predicted to occur from a failure of

FIG. 7. Localization of Plx1 changes during mitosis. (A) Late-blastula-stage embryos were fixed in methanol, and immunofluorescence staining was performed as previously described (30). α-Tubulin was detected with an anti-α-tubulin monoclonal antibody (Sigma) and visualized by Cy3-conjugated donkey anti-mouse IgG antibodies, and Plx1 was detected with anti-Plx1 antibodies and visualized by Cy2-conjugated donkey anti-rabbit IgG antibodies. Confocal microscopy was performed with an MRC-600 microscope (Bio-Rad). Bars, 10 μm. (B) Embryos were fixed as in panel A. Plx1 was visualized by Cy3-conjugated donkey anti-rabbit IgG antibodies and DNA was detected with SYTOX Green. Control IgG from immune sera that had been depleted of all Plx1-specific antibodies was used as a negative control (b).
cytokinesis, other defects, including a failure of chromosome segregation, could also give a similar phenotype. In addition, nuclei in a common cytoplasm might fuse together and/or continue to undergo rounds of DNA synthesis and division. Further work is needed to fully understand the basis of the cleavage arrest that results from the constitutive activity of Plk1.

In summary, the generation of a constitutively active form of Plk1 has revealed the importance of the enzyme at multiple stages of mitosis. The various functions in mitosis in which plk’s are implicated are correlated with the changes in the subcellular localization of Plk1 (Fig. 7). The domains of plk’s involved in localization changes are not clear, but it has been reported that mutants in the conserved polo box fail to localize to the midbody (20). Together with loss-of-function mutants, such as the N172A enzyme, elucidation of the mitotic functions of plk’s in a variety of organisms should be enhanced with constitutively active Plk.

ACKNOWLEDGMENTS

We thank Andrea Lewellyn for help with sectioning embryos and R. L. Erikson (Harvard University) for helpful discussions early in the course of this work. The baculovirus-infected S9 cells were produced in the tissue culture-monoclonal antibody core facility at the University of Colorado Cancer Center (P309CA46934). We thank Andrea Lewellyn for help with sectioning embryos and R. L. Erikson (Harvard University) for helpful discussions early in the order of cell cycle events. Science 245:1664–1672.

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