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POLO-LIKE KINASES IN CELL CYCLE CHECKPOINT CONTROL

Wei Dai, Xuan Huang, Qin Ruan

Division of Molecular Carcinogenesis, Department of Medicine, New York Medical College, Valhalla, NY 10595

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1. ABSTRACT

Recent studies from various eukaryotic model systems indicate that polo-like kinases (Plks) play an ever-increasing role in the regulation of cell cycle progression. Early genetic studies have demonstrated that Cdc5, a budding yeast counterpart of vertebrate Plks, is essential for mitosis. Mammalian Plks primarily localize to the microtubule organization center during interphase and undergo dramatic subcellular relocation during mitotic progression. Many key cell cycle regulators such as p53, Cdc25C, cyclin B, and components of the anaphase promoting complex are directly targeted by Plks. Although the exact mechanisms of action of these protein kinases *in vivo* remain to be elucidated, Plks appear to orchestrate various cell cycle checkpoints (intra-S phase, G_2/M transition, spindle assembly, and cytokinesis checkpoints) that protect cells against genetic instability during cell division.

1. INTRODUCTION

Reversible phosphorylation is a fundamental mechanism regulating cell cycle progression. To date, several families of protein kinases (e.g., cyclin-dependent kinases, polo-like kinases, and Aurora family of kinases) have been characterized that are important to the regulation of cell cycle progression. Recent studies have shown that polo-like kinases (Plks) are involved in controlling various cell cycle checkpoints.

Plks consist of a family of protein kinases sharing a common structural motif called polo-box (1). Polo or its homologues have been described in yeast (2), Caenorhabditis elegans (3), Drosophila melanogaster (4), Xenopus laevis (5,6), mouse (7), and human (1,8). The founding member of this family, polo, was originally identified in the fruit fly and shown to be a serine-threonine kinase that is required for mitosis (4). Mutations in the polo gene result in abnormal mitotic and meiotic division (9). The kinase activity of polo peaks cyclically at anaphase-telophase, and the protein also undergoes cell cycle-dependent changes in its intracellular distribution (10). CDC5 and Plo1 are structural as well as the functional homologues of polo in the budding yeast and fission yeast, respectively (11). Both genes are essential and loss of their function leads to mitotic arrest (2,12).

Vertebrate cells contain at least three proteins (Plk1, Plk2, and Plk3) that exhibit marked sequence homology to polo (7,13,14). Mammalian Sak, a protein serine/threonine kinase sharing significant homolog with Plk2 (Snk) in the catalytic domain, has been also described as a close relative of polo family kinase (15,16). The C. elegans genome contains three polo structural homologues (3,17). Interestingly, to date no additional gene products structurally homologous to polo have been identified in Drosophila. Given the lower evolutionary hierarchy of C. elegans compared to Drosophila, it is reasonable to predict that the fruit fly genome may contain additional Polo homologues.

3. INTRA-S PHASE CHECKPOINT

A possible role for Plks in intra-S-phase checkpoint response is implicated from several early studies. Expression of both mammalian Plk2 and Plk3 is rapid inducible by mitogen treatment (1,14). Induction of Plk3 mRNA by mitogens is protein synthesis-independent, suggesting that it is an immediate early gene product (1). Furthermore, microinjection of sense Plk1 mRNA, but not antisense RNA, into NIH3T3 cells that have been serum-starved results in incorporation of ^{3}H-thymidine (13). This suggests that Plk1 is required for DNA synthesis and overexpression of Plk1 appears to be sufficient for induction of DNA synthesis. During S phase, the genome is most susceptible to damage caused by environmental stresses or internal errors. Thus, it is natural that cells have

evolved mechanisms monitoring DNA damage, initiating repair processes if the damage is not extensive. Both Cdc5 in budding yeast and mammalian Plks are known to participate in the DNA damage response (18,19). Cdc5 is shown to play a primary role in controlling adaptation to DNA damage checkpoint in S. cerevisiae and the adaptation phenotype is abolished by Rad9 deletion (20). The electrophoretic mobility of Cdc5 in denaturing gels is affected by prior subjection of cells to DNA damage, and this modification is dependent on Mec1, Rad53 (a yeast Chk1 homolog), and Rad9 (18). In addition, a functionally defective Cdc5 mutant protein suppresses a Rad53 checkpoint defect, whereas overexpression of Cdc5 overrides checkpoint-induced cell cycle arrest (19), suggesting that Cdc5 acts downstream of Rad53.

Extensive research in the past decade has shown that the signaling pathways that underlie the cellular response to DNA damage (genotoxic stress) consist of sensors, signal transducers, and effectors [21]. Although the identities of the damage sensors remain unknown, the molecular entities responsible for transducing the damage signals to specific effectors are relatively well characterized. ATM (mutated in ataxia telangiectasia) and its homolog ATR (ATM related) function early in the signaling pathways and are central to the DNA damage response (21). Downstream targets (substrates) of ATM and ATR include the protein kinases Chk1 and Chk2. Recently, it has been shown that Plk1 activity is inhibited upon DNA damage; the DNA damage-induced inhibition appears to be mediated by prevention of its activation because expression of activation mutants of Plk1 can override the G_2/M arrest induced by DNA damage (22). Subsequent studies by this group reveal that DNA damage-induced inhibition of Plk1 is at least in part dependent on ATM or ATR because caffeine treatment prevents the inhibition of Plk1 in cells with DNA damage induced by IR or UV (23). In contrast, it has also been shown that Chk2 coimmunoprecipitates with Plk1 and that Plk1 overexpression enhances phosphorylation of Chk2 at T68 (24), a site primarily targeted by ATM and its phosphorylation is correlated with its activation (25). In addition, Plk1 phosphorylates recombinant Chk2 *in vitro* at the same site, suggestive of a positive involvement of this kinase in DNA damage response.

Plk2 (alternatively named Snk) and Plk3 (alternatively named Prk or Fnk) are also involved in DNA damage checkpoint activation pathway. Expression of PLK2 mRNA is rapidly induced in human thyroid cells upon X-ray irradiation; a radiation-responsive element has been identified as p53RE, a p53-binding homology element, in the basal promoter region of this gene (26). Similarly, Plk3 kinase activity is activated upon oxidative stress and DNA damage induced by ionizing-radiation mimetic drugs, and its activation is ATM-dependent (27,28). We have demonstrated that Plk3 interacts with and phosphorylates p53 and that it targets serine-20 of p53 *in vitro* (27). In response to DNA damage, the kinase activity of Plk3 was rapidly increased in an ATM-dependent manner (27,28). Peptide mapping as well as *in vitro* phosphorylation followed by immunoblot analysis with antibodies specific for phosphorylated forms of p53 also indicated that Plk3 phosphorylates p53 on physiologically relevant sites. Recombinant Plk1 also phosphorylates p53 but on residues that differ from those targeted by Plk3. Immunoprecipitation and "pull-down" assays have revealed that Plk3 physically interacts with p53 and that the extent of this interaction is increased in response to DNA damage (27). These results suggest that Plk3 functionally links DNA damage to the induction of cell cycle arrest or apoptosis. Recently, two independent studies report physical interaction between Plk3 with Chk2 (29,30) and functional connection between these two enzymes during DNA damage checkpoint activation. Plk3 phosphorylates Chk2 on a residue different from threonine 68. Plk3 appears to contribute to the full activation of Chk2 although ATM is necessary for phosphorylation and activation of Chk2 *in vivo* (29).

4. G_2/M CHECKPOINT

Mammalian Plks phosphorylate and regulate two key molecular players involved in controlling mitotic onset. Early work has shown that Plx1, a Xenopus orthologue of mammalian Plk1, regulate cyclin B/Cdc2, the activity of which is required for initiation of mitosis (31). Recombinant Plx1 is capable of phosphorylation of Cdc25, an activator of Cdc2, and stimulation of its activity *in vitro*; in addition, Cdc25 phosphorylated by Plx1 exhibits strong MPM-2 epitopes (32). The activation of Cdc25 by Plk1 is also demonstrated in a mammalian system. Human Plk1 protein immunoprecipitated from G_2/M-arrested cells directly phosphorylates Cdc25C; moreover, purified recombinant human Plk1 phosphorylates recombinant Cdc25C in a time- and concentration-dependent manner and this phosphorylation leads to the activation of the phosphatase (33). The exact molecular mechanism of activation of Cdc25C by Plk1 *in vivo* remains largely unknown. However, a recent study suggests that Plk1 may promotes the translocation of Cdc25C into the nucleus by phosphorylation of Serine-198 in a nuclear export signal sequence of human Cdc25C (34) because constitutively active Plk1 promotes the nuclear localization of this dual-specific protein phosphatase *in vivo* and because a mutant Cdc25C in which Serine-198 is replaced by alanine remains in the cytoplasm during prophase whereas wild-type Cdc25C accumulates in the nucleus. Together, Plk1 may positively regulate Cdc25C through both enhancing its phosphatase activity and promoting its nuclear localization at the mitotic transition.

Interestingly, Plk1 and its orthologue regulate the nuclear translocation of cyclin B, resulting in activation of Cdc2 as well (35). Plx1 appears to be the primary enzyme that phosphorylates serine-147 *in vitro* within the nuclear export signal sequence of cyclin B1 as antibody depletion abolishes the activity of Plx1 towards cyclin B1. Serine-147 appears to be involved in both the retention of cyclin B in the nucleus and the activity of Cdc2 because a phospho-specific antibody to this residue detects cyclin B1 only during G_2/M phase and because a mutant cyclin B1 with serines 133 and 147 replaced with alanines remains in the cytoplasm (35). A subsequent study demonstrates that

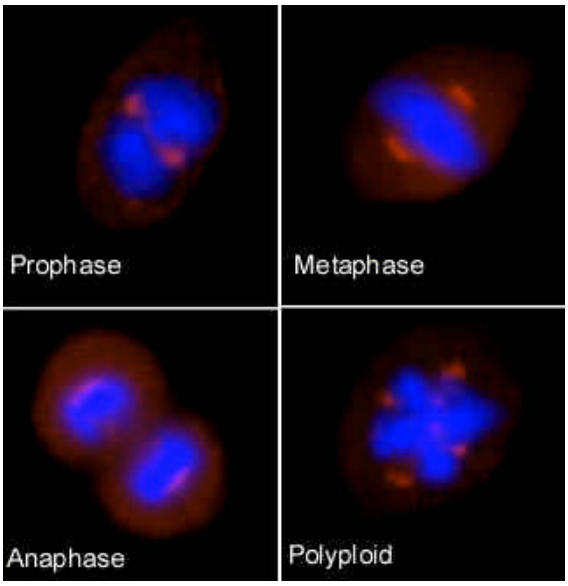

Figure 1. Subcellular localization of Plk3 during mitosis. Normal rat kidney cells were stained with a monoclonal antibody to Plk3 (red). DNA was stained with DAPI. Specific signals were detected using indirect immunofluorescent microscopy. Representative cells of various mitotic stages and a polyploidy cell were shown.

human cyclin B1 contains four major serine residues (serines 126, 128, 133, and 147), phosphorylation of which is important for its nuclear translocation (36). Moreover, Plk1 is primarily responsible for phosphorylation of serine-133 (36). However, there exists cooperativity between Plk1 and Erk2 in phosphorylation and activation of cyclin B1 (36).

Plk3, on the other hand, appears to be negatively involved in regulating Cdc25C activity. Our *in vitro* studies showed that His-tagged recombinant Plk3, but not its deletion mutant, phosphorylates GST-Cdc25C (37). Our subsequent studies confirmed the phosphorylation of Cdc25C by purified recombinant His6-Plk3, but not by a kinase-defective mutant His_6-$Plk3^{K52R}$ *in vitro* (38). In addition, phosphopeptide mapping showed that His_6-Plk3 phosphorylates His_6-Cdc25C at two sites *in vitro* and that the major phosphorylation site co-migrates with the one that is phosphorylated *in vivo* in asynchroneous cells; moreover, His_6-Plk3 strongly phosphorylates Cdc25C *in vitro* on serine-216 (38), a residue whose phosphorylation creates a binding site for 14-3-3, resulting in retention of this phosphatase in the cytoplasm (39,40). Given its activation by a variety of genotoxic stresses, Plk3 may play an essential role in mediating G_2/M arrest upon DNA damage.

5. SPINDLE ASSEMBLY CHECKPOINT

The accurate segregation of chromosomes during mitosis is a crucial cellular process that depends on the formation of intact bipolar spindles. Faithful transmission of chromosomes is at least partly regulated by the spindle assembly checkpoint, a mechanism preventing the cell from premature entering anaphase until all replicated and condensed chromosomes have attached to functional bipolar spindles. Early studies have demonstrated the importance of Plks in the formation of bipolar spindle (12,41,42). In fission yeast, either disruption of the Plo1 or its overexpression resulted in the formation of monopolar spindles as a consequence of the failure of the spindle pole body to complete either its duplication or separation (12). Vertebrate Plks are also involved in regulating bipolar spindle formation. In HeLa cells, injection of an antibody to Plk1 leads to a mitotic arrest with a monopolar spindle formed around a centrosome that is smaller than usual (41). The mechanism by which Plk1, or its orthologue, regulates mitotic spindle formation remains nebulous. However, it has been shown that Plx1 directly phosphorylates oncoprotein 18 (Op18, also termed stathmin), a microtubule-destabilizing protein, and thereby negatively regulates its activity (43). Plx1 extensively phosphorylates Op18 at the onset of mitosis, promoting microtubule stabilization and spindle assembly. Thus, Op18 may be one of those substrates, phosphorylation of which by Plx1 during mitosis plays a key role during mitotic progression.

Entry into anaphase requires the proteolytic destruction of the anaphase inhibitory protein securin by anaphase promoting complex (APC, an E3 ubiquitin-protein ligase), leading to the sister chromatid separation. Either the presence of unattached kinetochore or a loss of tension triggers the activation of spindle assembly checkpoint. We have recently observed that in normal rat kidney cells strong Plk3 signals are detected at the spindle poles during prophase and metaphase; the signals diminish significantly when the cell enters anaphase (Figure 1). We have also observed that Plk3 is activated rapidly upon microtubule disruption and that it interacts with BUBR1 (unpublished data), a major spindle checkpoint component. These observations suggest that Plk3 may play a part in spindle checkpoint response. Several other studies show that Plk1 is associated with APC components at the metaphase-anaphase transition, thus, indirectly regulating its ubiquitination activity (44-47). Mouse Plk1 specifically phosphorylates at least three components of APC (subunits Cdc16, Cdc27, and Tsg24) and activates APC *in vitro* (44). Recent studies have elucidated the molecular mechanism by which Plk1 and its yeast counterparts regulate sister chromatid separation. In S. cerevisiae, Cdc5 phosphorylates serine residues adjacent to the cleavage site of Scc1, a subunit of cohesion, and thereby enhances its cleavage by separase at the onset of anaphase (47). In fission yeast, Plo1 physically interacts with Cut23, a subunit of APC, through its noncatalytic domain; the interaction is compromised by Cut23 mutation, resulting in subsequent metaphase arrest; this phenotype is rescued by overexpression of Plo1 (46). Similar results are also obtained from Xenopus regarding the role of Plx1 in destabilization of the linkage of sister chromatids because when Plx1 is depleted from Xenopus egg extracts the release of cohesin during prophase is blocked; this blockage is more pronounced when both Plx1 and aurora B are simultaneously depleted (45), suggesting that these two mitotic kinases may cooperate to promote mitotic progression. Since these two kinases phosphorylate

different sets of substrates *in vitro* they may control the transition into anaphase through distinct mechanisms (45).

6. CYTOKINESIS (MITOTIC EXIT) CHECKPOINT

During mitosis, APC also targets cyclin B, whose degradation is essential for the exit from mitosis. It has been shown that the addition of catalytically inactive Plx1 mutant to M phase-arrested Xenopus egg extracts inhibits the proteolytic destruction of several APC targets, the inactivation of Cdc2 protein kinase activity, and the entry into interphase induced by Ca^{2+} (48). Destruction boxes have been identified in the N-terminus of Cdc5 but not in Plks of other species (8), indicating that at least some of Polo-like kinases may function as the activator as well as the target of APC. Interestingly, Plk1 also interacts with 20S and 26S proteasome subunits and phosphorylates 20S proteasome, leading to its enhanced proteolytic activity (49). Thus, Plk1 functions as a mitotic regulator of proteolytic activities through its effect on both APC and proteasomes. In addition, several recent studies reported that Cdc5 is part of the FEAR (Cdc **f**ourteen **e**arly **a**naphase **r**elease) network together with the separase Esp1 and the kinetochore-associated protein Slk19 and Spo12 (50). The FEAR network has been shown to initiate Cdc14 release from Cfi1/Net1 during early anaphase whereas the mitotic exit network (MEN) maintains Cdc14 in the released state during late anaphase and telophase (50). In fact, Cdc5 is capable of affecting the phosphorylation state of Net1, a nucleolar inhibitor of Cdc14, thus reducing its affinity with Cdc14 (51). Given that Cdc14 is required for the timely activation of MEN (50), Cdc14 release through the FEAR network is an important step for initiating mitotic exit.

Cytokinesis in animal cells is achieved by the formation of actin ring that contracts to divide cytoplasm and Plks appear to be also an important regulator of this process. Overexpression of wild-type Cdc5 or a catalytically inactive form results in the formation of multinucleated cells in budding yeast, which is apparently due to negative regulation of Swe1, a Wee1 kinase, by Cdc5 (52). In addition, a loss of S. pombe Plo1 function leads to the failure of septation both in the formation of an F-actin ring and in the deposition of septal material, suggesting that Plo1 function is required in the regulatory cascade that controls septation (12). The overexpression of Plo1 also induces the formation of multiple septa without nuclear division (12). A more detailed analysis on Plo1 indicated that it plays a role in the positioning of division sites by regulating Mid1p, a known gene product regulating cytokinesis in fission yeast (53). A recent study on cytokinetic actomyosin ring (CAR) has revealed that recruitment of Plo1 to the spindle pole body is substantially reduced in the presence of the microtubule depolymerizing agent thiabendazole, resulting in a delay of up to 90 minutes in CAR formation (54). Several lines of evidence also point to a predominance of cytokinesis defects in spermatogenesis in hypomorphic alleles of the Drosophila polo gene (55). Moreover, ectopic expression of wild-type mammalian PLK1 complements the cell division defect associated with the Cdc5-1 mutation in S. cerevisiae and the degree of complementation correlates closely with the Plk1 activity measured *in vitro*; expression of an activated Plk1 ($Plk1^{T210D}$) also induced a class of cells with unusually elongated buds which developed multiple septal structures (56).

Several mammalian Plks are found at the midbody region during later mitosis. It has been shown that the functional Polo box domain of Sak localizes the enzyme to the cleavage furrow during cytokinesis (57). It has also been demonstrated that EGFP-Plk3 fusion protein concentrates at the midbody and is associated with the cellular cortex (58), suggesting a functional role of Plk3 during mitotic exit. Our independent study confirms that ectopic expression of kinase active Plk3, but not the kinase-defective one, causes a defect in cytokinesis, eventually resulting in apoptosis (59). The mechanism by which mammalian Plks regulate cytokinesis remains largely unknown. However, Plk1 colocalizes with Pavarotti, a kinesin-related motor protein that is required for the organization of the central spindle, the formation of a contractile ring, and cytokinesis (60). This suggests that Plks may target motor proteins or their activity by phosphorylation during cellular exit from mitosis.

7. SUMMARY

Cell cycle checkpoints are surveillance mechanisms by which the cell ensures orderly progression during the cell cycle. Plks appear to orchestrate several important cell cycle checkpoints that protect cells against genetic instability during cell division. Although it remains unclear as to Plks' mechanisms of action especially in controlling intra-S phase checkpoint, spindle assembly checkpoint and cytokinesis checkpoint, an increasing amount of evidence suggests that protein kinases of the polo family are central players in the temporal and spatial coordination of cell cycle progression.

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Send correspondence to: Wei Dai, Ph.D., Molecular Carcinogenesis Division, Department of Medicine, New York Medical College, Valhalla, NY 10595, Tel: 914- 594-4724, Fax: 914-594-4726, E-mail: wei_dai@nymc.edu