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CDC6 controls dynamics of the first embryonic M-phase entry and progression via CDK1 inhibition

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ABSTRACT

CDC6 is essential for S-phase to initiate DNA replication. It also regulates M-phase exit by inhibiting the activity of the major M-phase protein kinase CDK1. Here we show that addition of recombinant CDC6 to Xenopus embryo cycling extract delays the M-phase entry and inhibits CDK1 during the whole M-phase. Down regulation of endogenous CDC6 accelerates the M-phase entry, abolishes the initial slow and progressive phase of histone H1 kinase activation and increases the level of CDK1 activity during the M-phase. All these effects are fully rescued by the addition of recombinant CDC6 to the extracts. Diminution of CDC6 level in mouse zygotes by two different methods results in accelerated entry into the first cell division showing physiological relevance of CDC6 in intact cells. Thus, CDC6 behaves as CDK1 inhibitor regulating not only the M-phase exit, but also the M-phase entry and progression via limiting the level of CDK1 activity. We propose a novel mechanism of M-phase entry controlled by CDC6 and counterbalancing cyclin B-mediated CDK1 activation. Thus, CDK1 activation proceeds with concomitant inhibition by CDC6, which tunes the timing of the M-phase entry during the embryonic cell cycle.

Introduction

CDC6, or Cell Division Cycle 6 protein, a member of the family of AAA+ATPases, is an essential and highly regulated component of the pre-replication complex required for loading MiniChromosome Maintenance (MCM) proteins onto the DNA (Perkins and Diffley, 1998; Weinreich et al., 1999). In the majority of organisms only one isoform of this protein is present. *Mus musculus* is the only organism, in which the presence of two isoforms of this protein was reported. Mouse isoforms of CDC6 are different variants of transcripts, which most probably appeared as result of alternative splicing. Variant 1 of *cdc6* gene transcript (# NM 011799.2 GenBank) was localized for the first time in mouse Embryonic Stem Cells (ES; Berger et al., 1999). Variant 2 was identified in the brain tissues of mouse embryo at 12.5 day of development (Strausberg et al., 2002). Variant 1 encodes isoform a, and variant 2 isoform b of Cdc6 protein. Both isoforms are highly homologous. In contrast to its function during DNA synthesis the role of CDC6 in mitosis is much less understood. It was shown that in yeast, CDC6 plays a dual role in the cell cycle control and it's over expression delays the M-phase entry (Bueno and Russell, 1992). CDC6 interacts with CDK1 upon M-phase exit; binding of CDC6 to CDK1 inhibits its kinase activity participating to exit from mitosis. Deletion of CDC6 lacking the CDK-interacting domain has no effect on DNA replication during S-phase, but slows down the mitotic exit (Elsasser et al., 1996; Calzada et al., 2001). However, these results were challenged by the experiments showing that in yeast exit from mitosis occurs in the absence of CDC6 (Archambault et al., 2003).

In HeLa cells CDC6 is phosphorylated during the M-phase by Polo-like kinase 1 (Plk1) (Yim and Erikson, 2010). CDC6 associates with Plk1 and localizes at the spindle pole in metaphase and central spindle in anaphase. The increase in CDC6 phosphorylation correlates with the increased level of Plk1; conversely CDC6 is hypophosphorylated in Plk1-depleted cells. Phosphorylation of CDC6 by Plk1 is required for its interaction with CDK1 and for CDK1 inhibition

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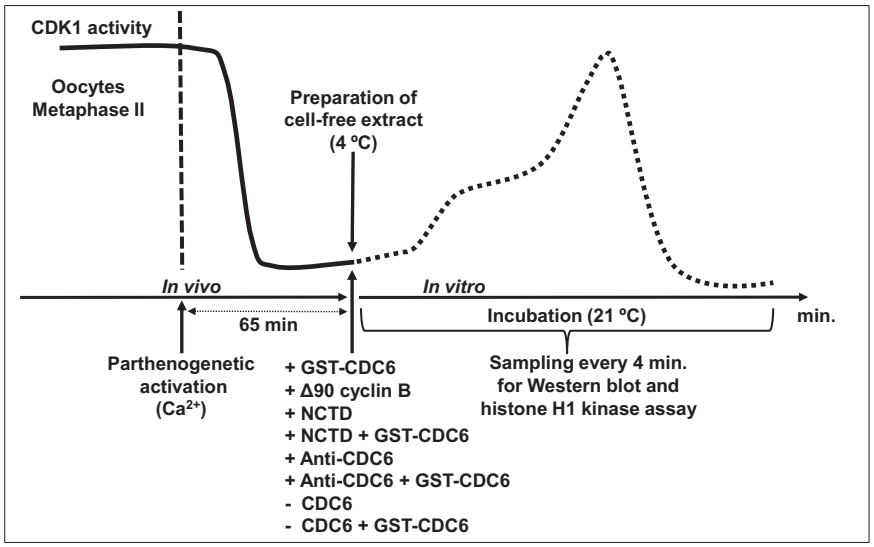

Fig. 1. Scheme of experiments.

necessary for separase activation and exit from mitosis (Yim and Erikson, 2010). Thus, CDC6 is clearly involved in CDK1 inactivation upon M-phase exit (ibid.) and, at least in HeLa cells, does not seem to be involved in regulation of earlier M-phase steps.

In mouse oocytes, CDC6 is necessary for the meiotic spindle formation and normal chromatin condensation (Anger et al., 2005). The involvement of CDC6 in spindle formation was also shown recently in *Xenopus oocytes* (Narasimhachar et al., 2012). The absence of CDC6 during oocyte maturation prevents DNA replication between two meiotic M-phases and its accumulation in MII-arrested oocytes is required for their competence to replicate DNA (Lemaitre et al., 2002; Whitmire et al., 2002). Thus, CDC6 is clearly involved in M-phase regulation in *Xenopus laevis*. In search for M-phase regulators in *X. laevis* we performed a proteomic screen to identify CDK1 partners during MII-arrest and upon oocyte activation and we found that CDC6 is associated with CDK1 during MII, when CDK1 remains stable (Marteil et al., 2012). This suggested to us that CDC6 might interact with CDK1 not only upon its inactivation during the M-phase exit, but also during other steps of the cell division. Thus, we asked whether CDC6 is involved in regulation of CDK1 during early M-phase stages, and in temporal regulation of mitotic divisions. In this study we prove the mitotic role of CDC6 as a CDK1 inhibitor in cell-free Xenopus embryo extracts. We show also that it regulates M-phase entry during the first embryonic division of mouse zygotes.

Material and methods

Xenopus egg collection and activation

X. laevis females were purchased from NASCO (Fort Atkinson, WI, USA). Females were injected subcutaneously with human chorionic gondotrophin (500 IU per female; Chorulon, Intervet, Netherlands) and kept overnight at 21 °C in 110 mM NaCl. Metaphase-arrested *Xenopus* eggs, collected from overnight spawning after HCG injection, were dejellied with 2% L-cysteine pH 7.81 in XB buffer (100 mM KCl, 1 mM MgCl2, 50 mM CaCl2, 10 mM HEPES, and 50 mM sucrose pH 7.6). Dejellied eggs were washed in XB, activated with calcium ionophore A23187 at 0.5 mg/ml until cortical contraction was observed, and then extensively washed in XB.

Xenopus cell free extracts

Cytoplasmic extracts from calcium ionophore-activated embryos before the first embryonic mitosis were prepared according to Murray (1991) with modifications made in our laboratory (Chesnel et al., 2005). Embryos transferred into appropriate tubes (5 ml ultraclear™ centrifuge tubes; Beckman Coulter, Roissy, France) and incubated at 4 °C. 0.5 ml XB buffer was then added containing protease inhibitors (10 μg/ml of each aprotinin, leupeptin, pepstatin, chymostatin, and 0.1 mM AEBSF) with 25 mg/ml cytochalasin D. Embryos were subjected to three consecutive centrifugations: a short spin to remove XB excess, a crushing spin 10,000*g* at 4 °C for 10 min, and a clarification 10,000*g* spin of the supernatant at 4 °C for 10 min. The supernatant was then supplemented with drugs or recombinant proteins and reincubated at 21 °C. Every 4 min, aliquots were taken out and directly mixed with Laemmli buffer for Western blot analyses. All aliquots were stored at −80 °C. For details of the experimental procedure see Fig. 1 in Results section.

Expression and purification of recombinant XlCDC6

Complementary DNA encoding wild-type *X. laevis* CDC6 was amplified by Polymerase Chain Reaction (PCR) and subcloned between Sal I/Not I restriction site pGEX-4T3 expression vector (plasmid pGEX). Wild-type XCDC6 was produced as glutathione *S*-transferase (GST) fusion proteins in bacteria (Furstenthal et al., 2001). *Escherichia coli* bacteria of BL21 strain were transformed in Lysogeny broth (LB) medium using plasmid pGEX-XCDC6 DNA or pGEX DNA alone. Bacteria membrane was destabilized by thermal shock to allow the plasmid incorporation. After incubation at 37 °C for 30 min, bacterial culture was added to LB agar medium containing ampicillin (0.1%) and then incubated over night at 37 °C. Resulting single bacterial colony was added to 5 ml of liquid medium LB/ampicillin 0.1% and incubated over night at 37 °C with constant agitation. 1 ml of liquid culture (BL21 pGEX and pGEX-XCDC6) was then added to 100 ml of medium LB/ampicillin 0.1% and incubated at 37 °C until reaching an optical density (at 600 nm wavelength) between 0.6 and 0.8. BL21 cells were grown and treated with isopropyl-1-thio-β galactoside 1 mM for 4 h at 37 °C to induce expression of the recombinant protein (GST-XCDC6). The cells were harvested by centrifugation, resuspended in lysis buffer (10 mM sodium phosphate, pH 7.2, 0.5 M NaCl, 1 mM EGTA, 1 mM

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