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Proteomics reveals a switch in CDK1-associated proteins upon M-phase exit during the *Xenopus laevis* oocyte to embryo transition

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ABSTRACT

Cyclin-dependent kinase 1 (CDK1) is a major M-phase kinase which requires the binding to a regulatory protein, Cyclin B, to be active. CDK1/Cyclin B complex is called M-phase promoting factor (MPF) for its key role in controlling both meiotic and mitotic M-phase of the cell cycle. CDK1 inactivation is necessary for oocyte activation and initiation of embryo development. This complex process requires both Cyclin B polyubiquitination and proteosomal degradation via the ubiquitin-conjugation pathway, followed by the dephosphorylation of the monomeric CDK1 on Thr161. Previous proteomic analyses revealed a number of CDK1-associated proteins in human HeLa cells. It is, however, unknown whether specific partners are involved in CDK1 inactivation upon M-phase exit. To better understand CDK1 regulation during MII-arrest and oocyte activation, we immunoprecipitated (IPed) CDK1 together with its associated proteins from M-phase-arrested and M-phase-exiting *Xenopus laevis* oocytes. A mass spectrometry (MS) analysis revealed a number of new putative CDK1 partners. Most importantly, the composition of the CDK1-associated complex changed rapidly during M-phase exit. Additionally, an analysis of CDK1 complexes precipitated with beads covered with p9 protein, a fission yeast *suc1* homologue well known for its high affinity for CDKs, was performed to identify the most abundant proteins associated with CDK1. The screen was auto-validated by identification of: (i) two forms of CDK1: Cdc2A and B, (ii) a set of Cyclins B with clearly diminishing number of peptides identified upon M-phase exit, (iii) a number of known CDK1 substrates (e.g. peroxiredoxine) and partners (e.g. HSPA8, a member of the HSP70 family) both in IP and in p9 precipitated pellets. In IP samples we also identified chaperones, which can modulate CDK1 three-dimensional structure, as well as calcineurin, a protein necessary for successful oocyte activation. These results shed a new light on CDK1 regulation via a dynamic change in the composition of the protein complex upon M-phase exit and the oocyte to embryo transition.

1. Introduction

MPF (M-phase promoting factor) is the universal molecular regulator of M-phase progression (Masui and Markert, 1971). Identification of the molecular nature of MPF was possible due to the *Xenopus laevis* oocytes appropriateness for biochemical analysis. MPF is composed of a kinase, CDK1 (cyclin-dependent kinase 1), and a regulatory subunit, Cyclin B (Gautier et al., 1988; Lohka et al., 1988). CDK1/Cyclin B is associated with a third component of the complex, the p9 protein (Xe-p9 in *Xenopus*, Cks1 and Cks2 in mammals, orthologues of fission yeast Suc1 protein; van Zon et al., 2010). The activation of CDK1 triggers M-phase entry, whereas its inactivation is linked to M-phase exit (Labbe et al., 1989; Riabowol et al., 1989). In *Xenopus*, CDK1 is activated during oocyte maturation and inactivated upon fertilization triggering embryo development. The sperm entry or a parthenogenetic treatment triggers an increase in intracellular free Ca^{2+} concentration promoting CDK1 inactivation that allows MII exit and the beginning of embryo development. This inactivation is due to the dissociation of CDK1/Cyclin B complex, Cyclin B degradation and CDK1 Thr-161 dephosphorylation (Nishiyama et al., 2000; Chesnel et al., 2006, 2007). More precisely, the APC/C (anaphase-promoting complex/cyclosome)-dependent polyubiquitination of Cyclin B targets the fully active CDK1/Cyclin B complex to the 26S proteasome. The 19S regulatory particle of the proteasome unfolds and dissociates Cyclin B from CDK1 (Nishiyama et al., 2000; Chesnel et al., 2006). Cyclin B dissociation is the earliest

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and indispensable step of CDK1 inactivation. Once separated from CDK1, Cyclin B is degraded, whereas the kinase is dephosphorylated on Thr-161 by type 2C protein phosphatase (Chesnel et al., 2007). Both CDK1 Thr-161 dephosphorylation and Cyclin B degradation ensure the irreversibility of CDK1 inactivation. CDK1 inactivation requires its T-loop refolding, which closes the access of the kinase enzymatic active site. This closure may be mediated by chaperones, assuring the rapidity of CDK1 inactivation (reviewed in Kubiak and El Dika, 2011). Thus CDK1 inactivation may require modifications in association with different partners.

The increasing knowledge on CDK1 makes the hypothesis of a role of CDK1-specific inhibitors and/or chaperones in CDK1 inactivation very attractive. In mammals, two types of CDK inhibitors (CKIs) have been described (for review see De Clercq and Inze, 2006). The Ink4 family inhibits CDK4 and CDK6 by binding to CDK instead of Cyclin (McConnell et al., 1999; Parry et al., 1999), while Cip/Kip family members bind to and inhibit CDK/Cyclin complexes (Chen et al., 1995). In *Saccharomyces cerevisiae*, Cdc6 in cooperation with Sic1 and Hct1 were postulated to participate in timely Cdc28 (CDK1-homologue) inhibition (Calzada et al., 2001). This function has been recently suggested by Yim and Erikson (2010) in HeLa cells, but has never been shown in developmentally regulated processes or in *Xenopus*. However, the CDK1 inhibitory role of Cdc6 remains controversial even in yeast (Archambault et al., 2003). No other CKI has ever been shown to regulate mitotic CDK1 activity, and we wondered whether such regulators could exist during M-phase and participate in the regulation of CDK1 regulation in *Xenopus*.

Our goal in the present study was to identify CDK1 interactors during M-phase-arrest and upon oocyte activation leading to the entry into the first embryonic interphase. For several reasons, this transition in *X. laevis* oocytes provides a unique opportunity for the biochemical study of putative changes in the composition of the CDK1 complex. First, the transition from active to inactive CDK1 is easily inducible and highly synchronous. Second, *X. laevis* oocyte cytoplasm is abundant and rich in proteins (Nishiyama et al., 2000; Chesnel et al., 2006, 2007). The feasibility of proteomic analysis of such oocytes and embryos was already demonstrated by our previous MS study of ubiquitinated *X. laevis* proteins (Bazile et al., 2008). Our results show that the composition of the CDK1 complex is indeed modified during CDK1 inactivation and consequently these data open new avenues for studying the function of so far unknown protein associations with this major M-phase regulator.

2. Materials and methods

2.1. Egg collection and activation

X. laevis females were purchased from NASCO (Fort Atkinson, WI). Females were subcutaneously injected with human chorionic gonadotropin (hCG, 500 IU/female). Unfertilized eggs (UFEs) were collected and washed with F1 buffer (31.25 mM NaCl, 1.75 mM KCl, 60 μM $MgCl_2$, 2 mM $NaHCO_3$, 10 mM Hepes, 0.25 mM $CaCl_2$, pH 7.6). UFEs were dejellied with 2% L-cysteine in F1 buffer, pH 7.8. Aliquots of 200 UFEs for both CDK1 IP and p9 precipitation and of 20 UFEs for Western blotting analysis were made and frozen in liquid nitrogen. Eggs were activated using 0.5 μg/mL calcium ionophore A23187 for 90 s. Aliquots of 200 activated UFEs were taken out about 7 min after ionophore treatment for both CDK1 IP and p9 precipitation, and aliquots of 20 eggs were taken out at 3, 7, 9, 15 and 30 min after treatment for Western blotting analysis.

2.2. Samples preparation for Western blotting

Eggs were homogenized in MPF-stabilizing buffer (80 mM β-glycerophosphate, 50 mM NaF, 20 mM EGTA, 20 mM Hepes, 15 mM $MgCl_2$, 1 mM DTT, pH 7.5) with mixture of protease inhibitors (1 mM AEBSF and 10 μg/mL of aprotinin, leupeptin, pepstatin) and 10 μg/mL of sodium orthovanadate and centrifuged (10,000 × *g*, 15 min, 4 °C). The egg extract was mixed with Laemmli buffer (Laemmli, 1970), heated at 85 °C and stored at −20 °C.

2.3. CDK1 immunoprecipitation

To IP CDK1 during M-phase and M-phase exit, we used protein extracts of unfertilized (200 UFE) and activated eggs (200 eggs at 7 min post activation). Affi-Prep protein A beads were washed three times with TBS–Triton X100 (50 mM Tris–HCl, 150 mM NaCl, 0.01% Triton X100, pH 7.5). The beads were then pre-equilibrated with or without (for IP negative control and for pre-clearing step) rabbit polyclonal antibody raised against *Xl*CDK1 C-terminal peptide (a gift from T. Lorca, CRBM, Montpellier, France), overnight at 4 °C in TBS–Triton X100 (50 mM Tris–HCl, 150 mM NaCl, 0.01% Triton X100, pH 7.5), supplemented with proteases inhibitors and sodium orthovanadate (as above). We selected this antibody because it was previously successfully used to specifically immunoprecipitate CDK1 from *X. laevis* oocytes (Krasinska et al., 2008). Fifteen hours later, aliquots of 200 eggs were homogenized in 1 mL of immunoprecipitation buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 50 mM NaF, 50 mM β-glycerophosphate, 0.2% NP-40) and centrifuged at 10,000 × *g* for 15 min at 4 °C. Protein extracts were centrifuged again at 10,000 × *g* for 15 min at 4 °C. Volumes of protein extracts were adjusted to 2 mL with IP buffer containing protease inhibitors, and a 15 μL aliquot was collected for Western blotting analysis ("total fraction"). After three brief washes with IP buffer, naked beads (200 μL per protein extract) were incubated with protein extracts and the mixture was agitated for 2 h at 4 °C to pre-clear the extract. The mixture of naked beads and egg extracts was then centrifuged at 4000 rpm, 3 min, 4 °C. The supernatants were mixed with 100 μL of naked beads (IP negative control) or of Affi-Prep protein A beads cross linked to CDK1 antibodies (dimethyl pimelimidate dihydrochloride was used as cross linking agent) previously washed twice with homogenizing buffer and agitated for 2.5 h at 4 °C. After centrifugation (1300 × *g*, 3 min, 4 °C), the supernatants were kept for a second round of IP, but an aliquot of 15 μL for each supernatant was conserved for Western blotting analysis ("unbound fraction"). The pellets were washed three times with 1.5 mL of TBS–Triton X100 with protease inhibitors. Proteins were eluted from beads using 100 μL of 100 mM glycine, pH 2.0. The eluted proteins were frozen at −20 °C and the beads were eluted a second time with glycine and washed three times with TBS–Triton X100 and two times with IP buffer. The additional round of IP was performed as previously described by incubating the supernatant of the first round with the beads. The two aliquots of eluted proteins were pooled and neutralized with 1 M Tris–HCl, pH 9.2. Laemmli buffer was added and the samples were heated at 85 °C for 5 min. A small aliquot was retained for Western blot analysis (bound fraction) and the remaining was analyzed by mass spectrometry.

2.4. CDK1 precipitation with p9 beads

To precipitate CDK1 with p9 beads during M-phase and M-phase exit, we used protein extracts made from unfertilized (200 UFE) and activated eggs (200 eggs taken out 7 min after activation). p9-Sepharose beads were a kind gift from L. Meijer and O. Lozach (Marine Station, Roscoff, France; Vogel et al., 2002).