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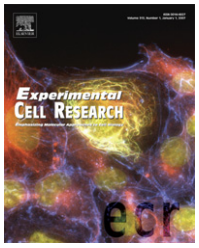

Research Article

Regulation of multiple cell cycle events by Cdc14 homologues in vertebrates

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ABSTRACT

Whereas early cytokinesis events have been relatively well studied, little is known about its final stage, abscission. The Cdc14 phosphatase is involved in the regulation of multiple cell cycle events, and in all systems studied Cdc14 misexpression leads to cytokinesis defects. In this work, we have cloned two *CDC14* cDNA from *Xenopus*, including a previously unreported *CDC14B* homologue. We use *Xenopus* and human cell lines and demonstrate that localization of Cdc14 proteins is independent of both cell-type and species specificity. Ectopically expressed XCdc14A is centrosomal in interphase and localizes to the midbody in cytokinesis. By using XCdc14A misregulation, we confirm its control over different cell cycle events and unravel new functions during abscission. XCdc14A regulates the G1/S and G2/M transitions. We show that Cdc25 is an *in vitro* substrate for XCdc14A and might be its target at the G2/M transition. Upregulated wild-type or phosphatase-dead XCdc14A arrest cells in a late stage of cytokinesis, connected by thin cytoplasmic bridges. It does not interfere with central spindle formation, nor with the relocalization of passenger protein and centralspindlin complexes to the midbody. We demonstrate that XCdc14A upregulation prevents targeting of exocyst and SNARE complexes to the midbody, both essential for abscission to occur.

Introduction

The molecular events that couple mitotic exit with cytokinesis have been best characterized in yeast. The mitotic exit network (MEN) in budding yeast [1] and septation initiation network (SIN) in fission yeast are homologous signaling pathways with highly conserved components [reviewed in 2]. At the base of these two pathways, however, one key downstream component is found: the dual specificity phosphatase, Cdc14p in *S. cerevisae* and Flp1 in *S. pombe*. The activation of Cdc14p in late anaphase is required for mitotic exit and it promotes cyclin dependent kinase inactivation through APC/Cdh1 regulation and Sic1 accumulation [1,3]. In contrast, although Flp1 can functionally complement the loss of Cdc14p, the SIN pathway is not required for mitotic exit *per se*. Instead, it induces a G2/M arrest as part of a cytokinesis checkpoint [4,5].

Although potential homologues of MEN components have been identified in higher eukaryotes, their functions and relationship with potential MEN-like regulatory pathways is still a matter of debate. In humans, two Cdc14 isoforms, hCDC14

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Abbreviations: MT, microtubule; GLU, detyrosinated tubulin; WT, wild-type; PD, phosphatase-dead; RT, room temperature
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A and hCDC14B, have been described [6,7]. Similarly to Cdc14p, hCDC14A can dephosphorylate Cdh1 *in vitro* and thus might promote activation of APC in late anaphase [8]. And, similarly to Flp1, hCDC14A and hCDC14B might regulate the activity of CDK1-cyclin B through Cdc25 phosphatase at the G2/M transition [9]. Deregulation of hCDC14A levels revealed its role in regulation of the centrosome cycle [6,7]. Finally, RNAi depletions of *C. elegans* Cdc14, CeCdc14, although leading to contradictory results, suggest a role for the phosphatase in cytokinesis as well as preventing extra cell divisions during quiescence [10,11]. Interestingly, whereas Gruneberg et al. show that CeCdc14 depletion leads to complete loss of central spindle and cytokinesis defect, Saito et al. demonstrate yet different consequences and a role for CeCdc14 in G1 to ensure cell cycle arrest. The discrepancies between the two studies have been ascribed to strain and RNAi concentration differences [12].

The complexity of the observed phenotypes might well correspond to involvement of Cdc14 in multiple regulatory events throughout the cell cycle. Some controversy over subcellular localization of human Cdc14 homologues exists, and the studies of phenotypes due to overexpression/depletion have not yet clearly identified Cdc14 functions. Nevertheless, a common feature of all Cdc14 proteins studied to date is the tight regulation of their cellular localization, which may also control their activity. In yeast, Cdc14 is sequestered inside the nucleolus and released in a regulated manner [13–16]. In *C. elegans*, CeCdc14 localizes to the central spindle and the midbody [10]. In humans, hCDC14B is found in nucleoli during interphase, becoming diffuse throughout the cell at mitosis entry. hCDC14A localizes to the centrosome in interphase and is lost from this structure in early mitosis [6,7]. However, further studies showed that both hCDC14A and hCDC14B could localize to the midbody [17,18], which was not described in previous studies.

Another feature of Cdc14 in all systems studied is its regulation of cytokinesis. CeCdc14 colocalizes at the central spindle with a component of the centralspindlin complex, the mitotic kinesin MKLP1/ZEN-4 [19], which it activates by dephosphorylation. In parallel, coordination of chromosome segregation and cytokinesis is assured by relocation of chromosomal passengers (including INCENP, Aurora B, Survivin, and Borealin) from metaphase kinetochores to the anaphase central spindle. While Cdc14p mediated dephosphorylation of yeast INCENP, sli5, is required for this translocation at the onset of anaphase [20], a similar role for Cdc14 in higher eukaryotes has not been investigated. Human CDC14A has recently been observed at the central spindle [17], a localization that would be consistent with its proposed regulation of potential substrates, MKLP1 and INCENP.

In *Xenopus*, Kaiser et al. [21] identified two Cdc14A paralogues but no Cdc14B homologue. Microinjection of *Xenopus* embryos with antibodies against XCdc14 interfered with the cell cycle, although the phenotype was not characterized. Given the still incomplete data on the involvement of Cdc14 in the control of cell cycle events, the different localizations ascribed to human CDC14A and B, and the unidentified cell cycle phenotype in *Xenopus*, in this report we attempt to better define the impact of vertebrate Cdc14 on cell cycle progression. To do so, we use a mixed *Xenopus*/ HeLa heterologous cell system, to enable study of functional conservation. We identify two *Xenopus* paralogues, XCdc14A and XCdc14B. We demonstrate that ectopically expressed XCdc14A is centrosomal during interphase while at the end of mitosis it is recruited to the midbody. Ectopic XCdc14A expression interferes with three important stages of cell cycle progression, the G1/S and G2/M transitions, and a late stage of cytokinesis, the abscission. We provide evidence that XCdc14A can prevent the G2/M transition by dephosphorylating Cdc25. During cytokinesis, it prevents the recruitment of SNARE/exocyst complexes, required for abscission to occur.

Materials and methods

cDNA cloning, immunization procedures, protein purification and antibodies

Database (http://www.ncbi.nlm.nih.gov) searches were used to identify Expressed Sequence Tag (EST) encoding protein homologous to metazoan *Cdc14* (GenBank accession nos. BG018336 and BJ08353). cDNAs were obtained from either Open Biosystems, USA or the *Xenopus laevis* cDNA ressource. Sequence analysis with DNA Strider software revealed that they encode two different proteins related to Cdc14, which we called XCdc14A and XCdc14B. These sequences have been deposited in GenBank (GenBank accession nos. AAV66581 and AAV66582, respectively).

For antibody production, PCR amplified XCdc14A (aa 1-489) and B ORF were subcloned into pET100d using a Topo cloning process (Invitrogen) and the 6His-tagged full-length XCdc14A and B were expressed in *E. coli* and purified over TALON affinity resin according to manufacturer instruction (BD Biosciences). For in vitro transcription/translation, the Cdc14A ORF was PCR-amplified with primers containing an XmaI site, with 6His sequence included in the reverse primer, and subcloned into SmaI site of pCS2 vector. XCdc14A wild type and phosphatase dead form were translated in rabbit reticulocyte lysate (TNT Sp6 Quick Master Mix; Promega) in the presence of [35S] methionine, according to the manufacturer's instructions.

For GFP expression studies, full-length wild type XCdc14A cDNA was subcloned into the XmaI site of pEGFP-C1 vector (CLONTECH). A point mutation in XCdc14A (phosphatase-dead mutant C276S) was generated using QuickChange site directed mutagenesis kit according to the manufacturer's protocol (Stratagene). All constructs were confirmed by DNA sequencing.

Xenopus egg extracts and in vitro kinase and phosphatase assays

Interphase egg extracts were prepared as described [22], snap-frozen in liquid nitrogen and stored at −80 °C. For G2/M transition studies, sperm chromatin (1000 sperm heads/μl) was added to interphase extract (40 μl) supplemented with gfp-NLS-GST construct, and incubated at RT for 20 minutes. Then 1,5 μl in vitro translated XCdc14A (WT or PD) or reticulocyte lysate (RL) was added, and incubated for 70 min. Entry into mitosis was induced with recombinant GST-tagged Δ90 cyclin B. For assessment of XCdc25 phosphorylation status, samples of extract (1 μl) were frozen in liquid nitrogen. For phosphatase assay, approximately 2 μg of bacterially produced GST-hCDC25C (clone gift from M. Morris) was phosphorylated by