Developmental Biology 341 (2010) 205–212

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Recruitment of *Orc6l*, a dormant maternal mRNA in mouse oocytes, is essential for DNA replication in 1-cell embryos

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ARTICLE INFO

Article history:
Received for publication 20 November 2009
Revised 18 February 2010
Accepted 19 February 2010
Available online 26 February 2010

Keywords:
DNA replication
Dormant maternal mRNA
Cytoplasmic polyadenylation element
Mouse oocyte
Mouse 1-cell embryo

ABSTRACT

Mouse oocytes acquire the ability to replicate DNA during meiotic maturation, presumably to ensure that DNA replication does not occur precociously between MI and MII and only after fertilization. Acquisition of DNA replication competence requires protein synthesis, but the identity of the proteins required for DNA replication is poorly described. In *Xenopus*, the only component missing for DNA replication competence is CDC6, which is synthesized from a dormant maternal mRNA recruited during oocyte maturation, and a similar situation also occurs during mouse oocyte maturation. We report that ORC6L is another component required for acquisition of DNA replication competence that is absent in mouse oocytes. The dormant maternal *Orc6l* mRNA is recruited during maturation via a CPE present in its 3′ UTR. RNAi-mediated ablation of maternal *Orc6l* mRNA prevents the maturation-associated increase in ORC6L protein and inhibits DNA replication in 1-cell embryos. These results suggest that mammalian oocytes have more complex mechanisms to establish DNA replication competence when compared to their *Xenopus* counterparts.

Introduction

Somatic cells have developed sophisticated mechanisms to regulate assembly of pre-replication complexes (preRC), license the ability of an origin of replication (ORI) to initiate DNA synthesis, and prevent re-replication of DNA (DePamphilis, 2003, 2005; DePamphilis et al., 2006). During the M to G1 transition, preRCs are assembled, which entails the assembly of an ORI that is composed of ORC1–6 at many sites in the genome. CDC6 and CDT1 are next recruited to these ORC:chromatin sites and in turn recruit an MCM complex composed of MCM2–7, a DNA helicase. Recruitment of MCM is termed replication licensing because the origin recognition complex (ORC) is now capable of supporting DNA replication. Licensing occurs in an environment of low cyclin-dependent protein kinase (CDK) activity. DNA synthesis is initiated by the further addition of MCM10 and the action of protein kinases, including CDKs, namely, CDK2 associated with either cyclin A or E. Once DNA replication has initiated following recruitment of CDC45 that in turn recruits DNA polymerase-α and DNA primase, re-replication of DNA is prevented by CDKs inhibiting the function of ORCs, CDC6, CDT1 and MCM, thus linking cell cycle progression with DNA replication.

In contrast to somatic cells, oocytes are arrested in the first meiotic prophase and have lost the ability to replicate DNA, ensuring that they maintain a 4C chromosome content. The ability to initiate DNA replication is acquired during oocyte maturation around MI and requires protein synthesis, but DNA replication does not occur until following fertilization or egg activation (Furuno et al., 1994). High levels of CDK1/CDC2A activity are required to maintain metaphase II arrest that in turn inhibits DNA replication (Furuno et al., 1994). The maturation-associated recruitment of *Mos* mRNA results in synthesis of MOS that is essential to maintain elevated levels of CDK1/CDC2A activity that in turn is required to maintain metaphase arrest (Colledge et al., 1994; Hashimoto et al., 1994). Inhibiting synthesis of MOS during maturation of *Xenopus* oocytes results in maturing oocytes entering interphase and replicating DNA soon after MI (Furuno et al., 1994). Entry into interphase is presumably because CDK1/CDC2A activity decreases, thereby relieving ORIs from their inhibited state as well as permitting formation of a nuclear membrane that is essential for DNA replication.

Xenopus oocytes contain all of the proteins required to assemble and license an ORI except for CDC6. Recruitment of *Cdc6* mRNA during maturation leads to the synthesis of CDC6 protein and restoration of the ability of the cytoplasm to support DNA replication (Lemaitre et al., 2002; Whitmire et al., 2002), i.e., synthesis of CDC6 can solely account for the protein synthesis requirement for acquisition of DNA replication competence during oocyte maturation. For example, blocking the maturation-associated increase in CDC6 protein by

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doi:10.1016/j.ydbio.2010.02.027

injecting anti-sense RNA directed at *Cdc6* mRNA in oocytes prior to maturation blocks DNA replication. DNA replication competence is restored, however, when CDC6 protein is also injected (Lemaitre et al., 2002).

Mouse oocytes also lack CDC6 protein and a maturation-associated recruitment of *Cdc6* mRNA results in CDC6 protein accumulation by MII (Anger et al., 2005; Lemaitre et al., 2004). A role for newly synthesized CDC6 in DNA replication following fertilization or egg activation could not be established because RNAi-mediated ablation of *Cdc6* mRNA inhibited oocyte maturation; oocytes underwent germinal vesicle breakdown but a spindle did not form and although chromosomes condensed, they did not form visible bivalents (Anger et al., 2005). Thus, whether recruitment of *Cdc6* mRNA is solely responsible for the maturation-associated acquisition of replication competence, as it is in *Xenopus* oocytes, remains an open question.

We had previously conducted microarray studies on mouse oocytes and 1-cell embryos and found that the relative abundance of several transcripts is increased in 1-cell embryos relative to oocytes, presumably due to polyadenylation because there is no transcription during this time and poly dT was used to prime the reverse transcription reaction (Zeng et al., 2004; Zeng and Schultz, 2005). We noted that the relative abundance of *Orc6l* mRNA was increased, making it another candidate whose recruitment would contribute to the maturation-associated acquisition of replication competence. We report here that ORC6L protein is undetectable in oocytes but present in metaphase II-arrested eggs due to a cytoplasmic polyadenylation element (CPE)-mediated recruitment of *Orc6l* mRNA. RNAi-mediated ablation of *Orc6l* mRNA prevents accumulation of ORC6L protein in metaphase II eggs and inhibits DNA replication following egg activation.

Materials and methods

Isolation and culture of oocytes and embryos

Full-grown, germinal vesicle intact oocytes (GV) were obtained from pregnant mare serum gonadotropin (PMSG)-primed, 6 week-old, CF-1 female mice (Harlan, Indianapolis, IN) and freed of attached cumulus cells as previously described (Schultz et al., 1983). Germinal vesicle breakdown (GVBD) was inhibited by adding 2.5 μM milrinone to the isolation and culture media (Tsafriri et al., 1996). The collection medium was bicarbonate-free minimal essential medium (Earle's salts) supplemented with 3 mg/ml of polyvinylpyrrolidone (PVP) and 25 mM Hepes (pH 7.3) (MEM-PVP). After collection, oocytes were cultured in CZB medium (Chatot et al., 1989) containing milrinone.

For isolation of metaphase II eggs (MII eggs) and 1-cell embryos, CF-1 female mice were superovulated with the injection of 5 IU of PMSG, followed 48 h later by 5 IU of human chorionic gonadotropin (hCG). MII eggs were collected 13–16 h post-hCG administration.

For generation of 1-cell embryos, after hCG injection the females were mated with B6D2F1/J male mice (Jackson Lab, Bar Harbor, ME) and embryos were collected 20–24 h after hCG. The cumulus cells were removed by a brief hyaluronidase treatment (3 mg/ml). One-cell embryos were cultured in 10-μl drops of KSOM supplemented with amino acids (KSOM+AA) under mineral oil (Ho et al., 1995). To generate parthenogenetic embryos, MII eggs were activated with 10 mM $SrCl_2$ in Ca^{2+}- and Mg^{2+}-free CZB for 2.5 h and further cultured in KSOM+AA, except in the experiment described in Fig. 2B in which the eggs were cultured in the presence of $SrCl_2$ for 6 h. When necessary, cycloheximide (20 μg/ml) or MG132 (10 μM) was added to the culture medium to inhibit protein synthesis or proteasome-mediated protein degradation, respectively. All oocytes and embryos were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Immunoblotting

Oocytes, eggs or embryos were directly lysed in SDS-PAGE sample buffer (100 mM Tris–HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), and stored at −20 °C until use. The samples were boiled for 5 min prior to being subjected to SDS-PAGE in a 12.5% gel. The proteins were then transferred to Immobilon-P (Millipore, Bedford, MA), and the membranes were blocked with 2–5% non-fatty milk in PBST (PBS with 0.2% Tween-20) for 1 h at room temperature, or overnight at 4 °C. After blocking, the membranes were washed twice in PBST for 10 min each and then incubated with an anti-ORC6L antibody (1:100 dilution in PBST; Cell Signaling Technology, Danvers, MA) overnight at 4 °C. Following incubation with the primary antibody, the membranes were washed 3 times in PBST for 15 min each and then incubated with horseradish peroxidase-conjugated secondary antibody (1:200,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. After washing in PBST for 3 times (15 min each), the membranes were developed using ECL Advance Western Blotting System (GE Healthcare, Piscataway, NY). As a loading control, membranes were stripped and reprobed with a mouse monoclonal anti β-tubulin antibody (cat # T4026, Sigma, St. Louis, MO) at a 1:10,000 dilution.

Immunofluorescence

Oocytes or eggs were fixed in 2% paraformaldehyde for 20 min at room temperature. The cells were permeabilized for 15 min in PBS containing 0.1% Triton X-100, and blocked in PBS containing 0.1% BSA and 0.01% Tween-20 (blocking solution); they were then incubated with the primary antibody for 1 h at room temperature (1:100 dilution in blocking solution). After three washes in blocking solution, the cells were incubated in the appropriate secondary antibody for 1 h (Alexa Fluor 488-conjugated anti-rat IgG was used [Invitrogen, Carlsbad, CA]). DNA was stained with 1.5 μg/ml propidium iodide (Invitrogen). The cells were then washed and mounted under a coverslip with gentle compression in VectaShield antibleaching solution (Vector Laboratories, Burlingame, CA). Fluorescence was detected on a Leica TCS SP laser-scanning confocal microscope.

Preparation of double-stranded RNA (dsRNA)

Total RNA was isolated from 30 GV oocytes using the Picopure RNA isolation kit (Arcturus, Sunnyvale, CA) according to the manufacturer's protocol. A reverse transcription (RT) reaction, primed with oligo dT, was performed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. PCR was performed using this oocyte cDNA to generate the templates for *in vitro* transcription. Two dsRNAs were used to target *Orc6l* mRNA. dsOrc6l corresponds to nucleotides 297 to 1085 (the entire coding region) and dsUTR, to nucleotides 1086 to 1653 (the entire 3′ UTR) of mouse *Orc6l* mRNA (accession number NM_019716.2). For amplification of the coding region of *Orc6l*, a pair of primers was designed based on the cDNA sequence. The sequence of the upstream primer was 5′-GCGATGGAGTCGGAGCTGG-3′ and the downstream primer was 5′-TGCTGTGGCTGTCTGAG-3′. These primers generated a PCR product that was 789 bp in length and corresponded to the entire coding region of *Orc6l*. For amplification of the 3′ UTR of *Orc6l*, the following primers were used: upstream primer: 5′-TTTCCATCT-CACTGCAGGCATG-3′, downstream primer: 5′-AATGTTTAAAAAA-TATTTATTAACTTAGGC-3′. These primers generated a PCR product that was 568 bp in length and corresponded to the entire 3′ UTR of *Orc6l*. Both PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI), the plasmids were digested and linearized with FspI and served as template for *in vitro* transcription. Sense