Full Length Research Paper

Adenosine diphosphate-ribosylation factor 1 controls spindle assembly during first meiotic division in bovine oocytes

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Accepted 25 May, 2012

Adenosine diphosphate-ribosylation factor 1 (ARF1) is a member of guanosine triphosphate (GTP)-binding proteins family associated with Golgi complexes. ARF1 regulated asymmetric cell division in female meiosis in mouse. However, little is known about its function in bovine oocyte meiosis. In the present study, we examined the localization, expression and functions of ARF1 during bovine oocyte meiotic maturation. Active form ARF1Q71L-Venus showed that ARF1 co-localized with α-tubulin on the spindle from the M I to the M II stage. Inhibition of ARF1 activity by microinjecting mRNA of a dominant negative mutant form of ARF1 (ARF1T31N) or ARF1 morpholino (ARF1 MO) into the germinal vesicle (GV) oocytes, and treating GV oocytes with brefeldin A (BFA), an inhibitor of Golgi-based membrane fusion led to abnormal spindle assembly and cytokinesis failure. On the contrary, microinjection of mRNA of a positive mutant form of ARF1 (ARF1Q71L) into GV oocytes had no effect on spindle assembly and the oocytes could undergo normal cytokinesis to generate one large egg with one small polar body. From the above, our results suggest that ARF1 plays an essential role in spindle assembly in bovine oocytes.

Key words: Adenosine diphosphate-ribosylation factor 1 (ARF1), spindle assembly, oocytes meiosis, cytokinesis, bovine.

INTRODUCTION

Female meiotic divisions are asymmetrical and generate a large oocyte and two small polar bodies in mammals, which is essential to preserve the maternal complement of resources necessary to support subsequent early development and maintain the chromosome number constant (Zheng and Dean, 2009). This asymmetry results from the anchoring of the meiotic spindle to the oocyte cortex and subsequent cortical reorganization (Leader et al., 2002; Maro and Verlhac, 2002; Verlhac et al., 2000). Several recent studies have reported that small GTPases, such as Ras-related nuclear protein (RAN) can prompt DNA-induced cortical polarization (Deng et al., 2007), Ras-related small GTP-binding protein (RAC) can regulate spindle stability and anchoring to the cortex (Cowan, 2007; Halet and Carroll, 2007), CDC42 can affect the migration of spindle and spindle microtubules in mouse oocytes (Ma et al., 2006), when these GTPases are inhibited, the affected mouse oocytes exhibit abnormal spindle assembly or fail cytokinesis.

In addition, small GTPase ADP-ribosylation factor 1 (ARF1) can control vesicular traffic from the endoplasmic reticulum (ER) to the Golgi apparatus and between successive Golgi compartments. ARF1T31N is a mutant which is likely to have a preferential affinity for GDP compared to the wild-type protein and may efficiently sequester an ARF1-specific guanine nucleotide exchange factor (ARF1-GEF). ARF1T31N can trigger a brefeldin A-like phenotype resulting in the redistribution of beta coatomer protein (β-COP) from Golgi membranes to the cytosol and the collapse of the Golgi into the ER.
Table 1. Details of primers used for gene cloning and mRNA detection.

| Gene   | GenBank accession no. | Primer sequence                      | Annealing temperature (°C) | Product size (bp) |
|--------|-----------------------|--------------------------------------|----------------------------|------------------|
| ARF1   | NM 176653             | F1: CACCATTCACCACATTGGGCTTC          | 60                         | 172              |
|        |                       | R1: ATTCACACGCTCTCTGATTCCG           |                            |                  |
| ARF1<sub>T31N</sub> | NM 176653             | F2: ATAAAGCTTTGTTGCCCATCGGCTG        | 60                         | 141              |
|        |                       | R3: GTTTGTCAGGATGTGTCCTTTCC          |                            |                  |
|        |                       | F3: GGAAGAAGACACATCCCTGTAACAAC       | 60                         | 480              |
|        |                       | R4: CGGATCAGGATTACACGCTGTTTCTGTTCC   |                            |                  |
| ARF1<sub>Q71L</sub>  | NM 176653             | F2: ATAAAGCTTTGTTGCCCATCGGCTG        | 60                         | 253              |
|        |                       | R4: GATCTTTGCTCAGCCACCGCTGTCC        |                            |                  |
|        |                       | F3: GAGCCTGGTGGTGGCGGACAAGATC        | 60                         | 360              |
|        |                       | R2: CGGATCCAGGATTACACGCTGTTTCTGGTTCC |                            |                  |

On the contrary, we provide evidence that ARF1 also plays an important role in regulating spindle assembly in bovine oocytes. By expressing the dominant negative mutant form ARF1<sub>T31N</sub> or microinjecting of ARF1 morpholino (ARF1 MO) in GV and treating GV oocytes with brefeldin A (BFA), we found that a large proportion of oocytes could not undergo cytokinesis and the spindles of oocytes were abnormal. These results demonstrate that ARF1 can affect bovine oocyte first meiosis, via the regulation of spindle assembly.

**MATERIALS AND METHODS**

**Bovine oocytes collection**

The ovaries were taken from slaughterhouse in Xi'an, and GV oocytes were collected from the ovaries by suction method. The GV oocytes enclosed by several layers of cumulus cells were selected and cultured in maturation medium (M199 supplemented with 10% fetal calf serum, 0.1 IU/mL HMG, 1.0 μg/mL Estradiol, 50 ng/mL EGF, 50 μg/mL Uracil, and ITS) for 24 h at 37°C under 5% CO₂.

**ARF1<sub>T31N</sub> and ARF1<sub>Q71L</sub> mutants construction and microinjection**

The mRNA of bovine oocytes was extracted with bovine cracking buffer liquid (5 mmol/L DTT, 20 IU/mL RNase inhibitor and 1% NP-40). Eukaryotic expression vector of pVenus was modified by inserting fluorescent protein Venus between HinIII and BamHI in pcDNA3.1 (Nagai et al., 1989). All primers are shown in Table 1. To detect the expression of ARF1 mRNA in bovine oocytes, PCR was performed with forward primer F1 and reverse primer R1. For construction of dominant negative mutant form of ARF1 (ARF1<sub>T31N</sub>), the first PCR was performed with forward primer F2 and reverse primer R3. The other PCR was performed with forward primer F3 and reverse primer R2. The italic lowercase represents the mutated base. Both PCR products were gel electrophoresis purified before use as templates for the second PCR with primers F2 and R2 to synthesize full length ARF1<sub>T31N</sub>. PCR product was digested with HinIII and BamHI and ligated into pVenus vector that had been digested with HinIII and BamHI.

For construction of the dominant active form of ARF1 (ARF1<sub>Q71L</sub>), reverse primer R4 and forward primer F4 were used. RNA synthesis in vitro was used mMESSAGE-mMACHINE (Ambion) and stored at -80°C. Bovine oocytes with several layers of cumulus cells were cultured in maturation medium containing 50 μM roscovitine (ROS) (Albarracin et al., 2005) to maintain them at the GV stage for 10 h. Subsequently equal quantities of mRNAs were microinjected into the cytoplasm of GV oocytes using Eppendorf Femtojet microinjector. The same amount of water was injected as the control. Each experiment consisted of three separate replicates and approximately 100 oocytes were injected in each group.

**ARF1 morpholino and control morpholino microinjection, BFA treatment**

Microscopic injection operation method refers to previous reports (Yun et al., 2009). Equal quantities of ARF1 MO (Table 2) were microinjected into the cytoplasm of GV bovine oocytes after treated with ROS for 10 h. The same amount of negative control MO was also injected as the control. The GV oocytes enclosed by several layers of cumulus cells were cultured in maturation medium containing 7 μg/mL BFA. Each experiment consisted of three separate replicates and approximately 100 oocytes were injected or treated in each group.

**Immunofluorescence staining**

All steps were performed at room temperature unless mentioned. The collected oocytes were fixed with fixative solution for at least 30 min, permeated for 20 min with 0.5% Triton X-100 in PBS, and all samples were incubated in the blocking solution (2% BSA, 2% skimmed milk powder, 0.15 mmol/L glycine and 0.05% Tween-20 in PBS) for 1 h, then oocytes were incubated with anti-α-tubulin first antibody with a dilution of 1: 500 (Beyotime) overnight at 4°C. After three times of washing with PBS containing 0.1% Tween-20 and 0.01% Triton X-100 for 5 min, the oocytes were incubated with Cy3 conjugated goat anti-mouse second antibody (Beyotime, dilution 1:500) for 2 h at 37°C for α-tubulin. After three times of washing with PBS containing 0.1% Tween-20 and 0.01% Triton X-100 for 5 min, the DNA was stained with 10 μg/mL Hoechst33342. Finally, the oocytes mounted on glass slides were observed with a fluorescence microscope and excitation wavelengths of 488, 550 and 343 nm.
Table 2. Details of morpholino oligo sequence used for mRNA deletion.

| Gene name   | Oligo sequence                  |
|-------------|---------------------------------|
| ARF1 MO     | TTGCAAAGATATTCCCCATGCTGCA       |
| Control MO  | ACGTCGTACCCCTTATAGAAACGTT       |

Figure 1. Expression of ARF1 mRNA is ubiquitously expressed at all the stages of bovine oocytes. Samples were collected after bovine oocytes was cultured for 0, 6, 12 and 24 h, corresponding to GV, GVBD, M I and M II stage, respectively. The length of ARF1 is 172 bp and that of β-actin is 445 bp.

Statistical analysis

Data (mean ± SE) were from at least three replicates per experiment and analyzed by Chi-square test using SPSS software. Difference at P < 0.05 was considered to be statistically significant and different superscripts indicate the statistical difference.

RESULTS

Subcellular localization and expression of ARF1 during bovine oocyte meiotic maturation

To investigate the role of ARF1 in bovine oocyte maturation, reverse transcriptase (RT)-PCR was performed to verify whether ARF1 is expressed in oocyte maturation. Our results indicate that ARF1 mRNA is ubiquitously expressed during all the stages of oocytes (Figure 1).

Since no specific antibodies are available to recognize the endogenous active form of ARF1 protein, so we expressed ARF1Q71L tagged with Venus, via mRNA microinjection, to determine its subcellular localization during bovine oocyte maturation. Active form ARF1Q71L-Venus was in heaps in the cytoplasm at the GV stage. Shortly after GVBD, ARF1 began to distribute uniformly in the cytoplasm until the M I spindle was formed. At A I and M II stage, ARF1 mainly localized to the spindle (Figure 2).

ARF1T31N expression or ARF1 deletion caused abnormal spindle assembly and M I arrest during bovine oocyte maturation

To assess the function of ARF1, mRNA encoding a dominant negative mutant form of ARF1 (ARF1T31N) or ARF1 MO was microinjected into the cytoplasm of GV bovine oocytes in the presence of roscovitine (ROS) which can inhibit spontaneous GVBD. The vitro maturation rate of GV oocytes which expressing ARF1T31N (25%, 33/132) (Figure 3B) or microinjected with ARF1 MO (24.4%, 30/123) (Figure 4B) were decreased significantly compared with control oocytes respectively (54.1%, 85/157; 51.9%, 83/160). Furthermore, immunofluorescence and statistical analysis showed that 71.2% (94/132) oocytes in the ARF1T31N expression (Figure 3C) and 72.4% (89/123) in the ARF1 MO group (Figure 4C) displayed abnormal spindles at M I and A I stages, but only 14% (22/157) and 15.6% (25/160) of oocytes in the control group showed similar phenotypes (Figures 3A and 4A).

BFA treating bovine oocytes disrupted spindle assembly, caused MI arrest

BFA can cause the inhibition of ER to Golgi transport, collapse of the Golgi into the ER (Donaldson et al., 1990; 1991), and ARF1 inactivation (Randazzo et al., 1993). Another drug, Exo1, can destroy the Golgi apparatus via a different mechanism, but do not directly inhibit ARF1-GDP/GTP exchange (Feng et al., 2003). We treated bovine oocytes with Exo1, all the Exo1-treated oocytes underwent normal meiosis and the spindle was normal. Then we excluded Golgi collapse as responsible for cytoplasmic division failure in bovine oocytes. Following we treated GV oocytes with BFA to investigate the role of BFA in bovine oocytes, we found that a part of oocytes were arrested in M I stage and the spindle was abnormal, and another part of oocytes could not form spindle, just show a mass of microtubules organization (Figure 5A), immunofluorescence and statistical analysis showed that 77.2% (112/145) oocytes in the BFA group displayed abnormal spindles, but only 14.9% (23/154) of oocytes in the control group showed similar phenotypes (Figure 5C), moreover, these oocytes showed cytokinesis failed, DNA did not attach to cortex and the vitro maturation rate of oocytes treated with BFA (20%, 29/145) decreased significantly compared to control oocytes (53.2%, 82/154) (Figure 5B). However alignment of the chromosomes was normal in oocytes treated with BFA. On the contrary, spindle of control oocytes displayed normally fusiform and chromosomes distributed in the vicinity of the cortex (Figure 5A).

DISCUSSION

During meiotic maturation of mammalian oocytes, oocytes undergo two successive meiotic divisions without an intermediate phase of DNA replication, so that haploid gametes are produced. However, the mechanisms involved are poorly understood. Previous studies show that inhibiting some small GTPases result in symmetric
Figure 2. Subcellular localization, expression of ARF1. Fluorescence microscopy showed active form of ARF1 protein ARF1^{Q71L} (green) and immunostaining of α-tublin (red) and DNA (blue) in bovine oocytes at GV, pre-M I, M II, A I and M II stage.

cell division or the failure of division in mouse oocytes. In the present study, we show that another small GTPase, ADP-ribosylation factor 1 (ARF1), plays an important role in cytoplasmic division during first meiotic division in bovine oocytes. By expressing the dominant negative mutant form of ARF1 (ARF1^{T31N}) to inhibit the activity of ARF1 or microinjecting ARF1 MO to interfere the expression of ARF1 mRNA in GV oocytes, we found that
Inhibition of ARF1 activity led to abnormal spindle assembly. After expression of ARF1$^{T31N}$, the bovine oocytes were incubated in maturation medium for 7 - 9 h. (A) Oocytes with abnormal spindle at the M I and M II stages. Fluorescence microscopy showed inactive form of ARF1 protein ARF1$^{T31N}$ (green) and immunostaining of α-tubulin (red) and DNA (blue) in oocytes. (B) Percentage of oocytes with different vitro maturation in the expression of ARF1$^{T31N}$ group (n = 132) and control group (n = 157). Data are presented as mean ± SE. Different superscripts indicate statistical difference (P<0.05). (C) Percentage of oocytes with abnormal spindles in the expression of ARF1$^{T31N}$ group (n = 132) and control group (n = 157). Data are presented as mean ± SE. Different superscripts indicate statistical difference (P < 0.05).

The spindles assembly of bovine oocytes were abnormal, most oocytes were arrested at M I stage and the oocytes could not undergo normal cell division. The faithful segregation of chromosomes ensures proper distribution of genetic material during cell division in mitosis and meiosis (Yin et al., 2008), and spindle microtubules play an important role in this event (Hoyt et al., 1991; Sluder, 1979). The first meiotic spindle migration depends on microfilaments and not microtubules in mouse oocytes (Longo and Chen, 1985; Van Blerkom and Bell, 1986).
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Figure 4. ARF1 deletion led to abnormal spindle assembly. After microinjection of ARF1 MO, the oocytes were incubated in maturation medium for 7-9 h. (A) Oocytes microinjected with ARF1 MO exhibited abnormal spindle morphology at the M I and M II stage. Double staining of α-tubulin (red) and DNA (blue). (B) Percentage of oocytes with different vitro maturation in the ARF1 MO microinjection group (n = 123) and control group (n = 160). Data are presented as mean ± SE. Different superscripts indicate statistical difference (P < 0.05). (C) Percentage of oocytes with abnormal spindles in the ARF1 MO microinjection group (n = 123) and control group (n = 160). Data are presented as mean±SE. Different superscripts indicate statistical difference (P < 0.05).

When microtubules are depolymerized with nocodazole, mouse meiotic chromosomes can still migrate to the cortex and induce the formation of a cortical domain on the cell cortex at a limited distance. In this case, ARF1T31N mainly localized at the spindle at M I stage, although the spindles were abnormal, all chromosomes could also
Figure 5. Oocytes treated with BFA arrested at the M I stage and the spindle was abnormal. (A) After treated with BFA, the spindle of oocytes was abnormal at M I stage or only microtubules distributed in the cytoplasm with no spindle at pre-M I stage. Fluorescence microscope showed immunostaining of α-tubulin (red) and DNA (blue) in oocytes treated with BFA arrested at the pre-M I and M II stage. (B) Percentage of oocytes with different vitro maturation in the BFA group (n = 145) and control group (n=154). Data are presented as mean ± SE. Different superscripts indicate statistical difference (P < 0.05). (C) Percentage of oocytes with abnormal spindles in the BFA group (n=145) and control group (n=154). Data are presented as mean ± SE. Different superscripts indicate statistical difference (P < 0.05).

attach to the bipolar spindle and were aligned at the equatorial plate, thus the presence of the spindle may influence the segregation of chromosome but was not required for normal chromosomes arrangement and the movement of the chromosomes to the cortex. In contrast to bovine oocytes, expressing ARF1T31N in GV mouse
oocytes led to the spindle diffused in the centre of the cytoplasm and the mouse oocytes generated two metaphase II (M II) oocytes of equal size (Wang et al., 2009).

When dominant positive mutant form of ARF1 (ARF1\(^{Q71L}\)) that maintained the activity of ARF1 was expressed in bovine oocytes, 18.2% (26/143) of oocytes displayed abnormal spindles, and 15.0% (20/133) of oocytes in the control group showed similar phenotypes which indicated no statistically significant differences. Additionally, the bovine oocytes can undergo normal first meiotic division and there was no influence on vitro maturation rate compared with the control group. ARF1\(^{Q71L}\) mainly localized at the spindle at A I and M II stages while mainly distributed in the cytoplasm before M I stage. We conclude that ARF1 regulates spindle assembly form. If ARF1 was not combined with GTP and inactive, the spindle of bovine oocytes will be abnormal and arrested at M I stage. Moreover, mouse oocytes can also undergo normal asymmetric division by expressing ARF1\(^{Q71L}\) at GV stage, however, active form ARF1\(^{Q71L}\) was found evenly distributed in the cytoplasm before and after GV breakdown (GVBD) and ARF1 was found concentrated at the contractile ring and co-localized with F-actin during polar body extrusion in mouse oocytes (Wang et al., 2009).

BFA-treated bovine oocytes also revealed abnormal spindle assembly and structure or no spindle but microtubules during first meiotic division and chromosomes could be normally aligned at the equatorial plate. However, mouse oocytes will generate metaphase II (M II) oocytes of equal size or inhibit cytokinesis when treated with BFA, and BFA treatment did not affect spindle assembly and structure but prevented the asymmetric spindle positioning and resulted in a symmetric cell division (Wang et al., 2008).

ARF proteins can function at different cellular membranes including the plasma membranes and the Golgi membranes (Donaldson and Jackson, 2000) and BFA can specifically prevents Golgi membranes catalysing the exchange of GTP onto ARF1 by inhibiting the interaction between ARF1 and ARF1-GEF (Donaldson et al., 1992; Peyroche et al., 1999). During mitosis of somatic cells, ARF1 activity is required for mitotic Golgi disassembly and mitotic Golgi fragmentation utilizes a different mechanism compared with the effect caused by BFA (Xiang et al., 2007).

Recently, ARF1 regulates MAPK activity independently from the MOS-MDK-MAPK pathway in mouse oocytes, expression of ARF1\(^{T31N}\) generated large polar bodies during meiosis which is similar to the phenomena observed in Mos knockout mice (Choi et al., 1996). MOS/MAPK pathway played essential role in spindle organization in mouse oocytes (Verlhac et al., 1994), and Mos was necessary for MAP kinase activation (Verlhac et al., 1996). In bovine oocytes, Mos was also required for MAPK activation (Fissore et al., 1996). We guess that ARF1 may finally regulate MAPK activity to affect spindle assembly, but whether or not ARF1 through MOS/MAPK pathway remained to be investigated.

All data show that ARF1 is essential for spindle assembly and plays a critical role in first meiotic division in bovine oocytes.

ACKNOWLEDGEMENTS

We are grateful to Keith Jones for his pVenus plasmid.

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