Protein kinase B (PKB/Akt) is required for the completion of meiosis in mouse oocytes

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Abstract

Akt, also known as protein kinase B, is implicated in many cellular processes. Akt is phosphorylated at two residues, Thr308 and Ser473. Thr308-phosphorylated Akt is present in pericentriolar materials, while localization of Ser473-phosphorylated Akt was similar to that of microtubules in metaphase oocytes. Spindles were shorter and aberrant in oocytes injected with Thr308- or Ser473-phosphorylated Akt antibodies. Specifically, Thr308- and Ser473-phosphorylated Akts function individually and are both necessary to assemble the metaphase II (MII) spindle. Moreover, the functions of Thr308- and Ser473-phosphorylated Akts differ in MII oocytes. Although oocytes exhibited second polar body (PB2) emission after the injection of a peptide for Thr308, the chromosomal alignment and microtubular organization were aberrant. In contrast, the injection of a peptide for Ser473 caused a failure of PB2 emission. These results suggest that Thr308- and Ser473-phosphorylated Akts are individually involved in fertilization to complete meiosis, including different roles (i.e., Ser473-phosphorylated Akts are involved in PB2 emission, whereas Thr308-phosphorylated Akts regulate the organization of microtubules).

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Introduction

The resumption of meiosis, morphologically identified by germinal vesicle breakdown (GVBD), is triggered in healthy follicles by a preovulatory luteinizing hormone (LH) surge. The progression of meiosis beyond GVBD involves chromosomal condensation and alignment of the metaphase I (MI) spindle, segregation of homologous chromosomes, emission of the first polar body (PB1), and formation of the MII spindle. GVBD and the progression of oocytes to MII are usually referred to as meiotic maturation.

Previously, we have demonstrated that phosphatidylinositol 3-kinase (PI3K) participates in follicle-stimulating hormone (FSH)-induced mouse meiotic maturation (Hoshino et al., 2004). LY294002, a specific inhibitor of PI3K, suppressed GVBD, PB1 emission, and cumulus expansion. LY294002 also decreased the amount of phosphorylated Akt in MI and MII oocytes (Hoshino et al., 2004). Akt, also known as protein kinase B, was identified as a serine–threonine kinase that functions downstream of PI3K (Hanada et al., 2004). Akt becomes phosphorylated at two residues, Thr308 and Ser473, and both residues are required for the full activation of Akt (Alessi et al., 1996). Our previous results demonstrated that the distribution of Ser473-phosphorylated Akt was similar to that of microtubules, whereas Thr308-phosphorylated Akt was present in the pericentriolar materials (PCM) in MI and MII oocytes (Hoshino et al., 2004). Furthermore, LY294002 decreased the amount of Thr308-phosphorylated Akt to very low to undetectable levels in MI and MII oocytes. Ser473-phosphorylated Akt exhibited an aberrant distribution and very low to undetectable levels of expression in LY294002-treated MI and MII oocytes, respectively. These observations of Akt in mouse oocytes suggest that Akt signaling via PI3K is important for oocyte maturation, and both Thr308-phosphorylated Akt and Ser473-phosphorylated Akt are associated with spindle function (Hoshino et al., 2004; Kalous et al., 2006).
Akt is a second messenger-regulated kinase that has been implicated in many crucial cellular processes such as glucose metabolism, transcription, cell proliferation, apoptosis, migration, and growth (Lawlor and Alessi, 2001). Deregulation of Akt activity contributes to cell transformation and diabetes (Nicholson and Anderson, 2002). Akt is well known for its antiapoptotic effects (Vanhaesebroeck and Alessi, 2000) and plays a role in the inhibition of entry into the S phase (Datta et al., 1999), while there is very limited information available regarding the G2/M phase of the cell cycle (Cheng et al., 1997; Kandel et al., 2002; Shitovleva et al., 2002; Wakefield et al., 2003). Although Akt is implicated in many crucial cellular processes, no reports have demonstrated that only one of those residues is phosphorylated under natural conditions. Notably, the difference in the localization of the two active forms could be related to their individual roles in meiosis. However, the role of Thr308-phosphorylated Akt in PCM and Ser473-phosphorylated Akt in the spindle is still unknown. The present investigation examined the functional role of Akt using a specific inhibitor, SH-6, during oocyte meiotic maturation. To determine the functions of phosphorylated Akt, peptides or antibodies against each form were microinjected into the cytoplasm of MI or MII oocytes. We demonstrate that individual Thr308- and Ser473-phosphorylated Akt activities are involved in fertilization to complete meiosis, including different roles. Here we provide evidence that Ser473-phosphorylated Akt activity is involved in PB2 emission while Thr308-phosphorylated Akt regulates the organization of microtubules for the completion of meiosis in mouse oocytes.

Materials and methods

**In vitro maturation, fertilization, and embryo culture**

Maturation of oocytes in vitro was performed as described previously (Hoshino et al., 2004). Oocytes at prometaphase I (PMI), MI, and MII were collected at 8, 10, and 18 h after the start of culture, respectively. In vitro fertilization and embryo culture were performed as described previously (Matsumoto et al., 2001). In brief, spermatozoa were preincubated for 2–3 h in human tubal fluid (HTF) medium to allow capacitation. The final concentration was 700 spermatozoa/μl. Oocytes were cultured in potassium simplex optimized medium (KSMOM) 4 h after insemination.

**Akt inhibitor**

For the inhibition of Akt, SH-6 (Calbiochem) was added to the culture medium. We prepared 50 mM stock solution of SH-6 in dimethyl sulfoxide and diluted it to the desired final concentration in culture medium.

**Immunostaining of oocytes**

Immunolocalization of Akt was performed as previously described (Hoshino et al., 2004). Akt and phosphorylated Akt were detected using anti-Akt, anti-Thr308-phosphorylated Akt, or anti-Ser473-phosphorylated Akt (Cell Signaling Technology; 1:100) and Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes; 1:200). Lamin B was detected using anti-Lamin B (Santa Cruz Biotechnology; 1:200) and Alexa Fluor 488-conjugated anti-goat IgG (Molecular Probes; 1:200). Microtubules were detected using anti-α-tubulin (Sigma; 1:500) and Alexa Fluor 488-conjugated anti-mouse IgG (Jackson ImmunoResearch; 1:200) or Cy5-labeled anti-mouse IgG (Jackson ImmunoResearch; 1:200). Chromosomes were labeled with 10 μg/ml propidium iodide (Sigma).

The oocytes were then viewed using a Bio-Rad MRC-1024 confocal scanning laser microscope mounted on an Axioplan Zeiss microscope. Spindle length was measured using Motic Images Plus 2.0S (Shimadzu).

**Microinjection of peptides and antibodies**

The following phosphorylated Akt peptides were synthesized and purified by high-performance liquid chromatography (HPLC; Operon Biotechnologies): NH2-(pT)FCGTPPEYLAPCEOOH for Thr308 and NH2-FPQF(pS)SYS-COOH for Ser473. Thr308- and Ser473-phosphorylated Akt antibodies were concentrated and purified with a microcon (Millipore). Oocytes were microinjected into the cytoplasm with ~1 pl of the phosphorylated Akt inhibitory peptides (1 mM) or antibodies (2 μg/μl) with a micromanipulator (Beaunean et al., 2000).

**Immunoblotting**

Oocytes were collected and placed in 2× sodium dodecyl sulfate (SDS) sample buffer, 0.5 M Tris–HCl (pH 6.8), 10% 2-mercaptoethanol, and 20% glycerol. Lysates were separated by electrophoresis and transferred to Immobilon membranes (Millipore). Membranes were incubated with antibodies against Akt, Thr308-phosphorylated Akt, and Ser473-phosphorylated Akt (1:1,000) overnight at 4 °C. The detection of antigens was achieved with an ABC–PO system, and peroxidase activity was visualized using the DAB kit (Vector Laboratories).

**RT–PCR**

Total RNA extraction and DNA synthesis were performed using Cells-to-cDNA™ II (Ambion). PCR was performed using Ex Taq polymerase (TaKaRa). Each primer was designed according to published mouse cDNAs. For Akt1, the sense (5′-GCCAAAGTCCGCAAGAAGG-3′) and antisense (5′-CTGACCCGCGATGGACACAG-3′) generated a 182-bp fragment. For Akt2, the sense (5′-CTGCCCCGTAGCTACGTTAAG-3′) and antisense (5′-CGGGCTCCTCTTATACCCA-3′) generated a 176-bp fragment. For Akt3, the sense (5′-CCTACCAACTCCACCTGAC-3′) and antisense (5′-GAAAGTGCAGCTCCGGAGA-3′) generated a 181-bp fragment. For G3PDH, the sense (5′-CCACTCTTCCACTTCGATG-3′) and antisense (5′-GAGGGGAGATGCT-CAGTGGTTG-3′) generated a 226-bp fragment. The amplification conditions were as follows: 94 °C for 5 min; 35 cycles of 20 s each of denaturation at 94 °C, annealing at 58 °C (for Akt2, 56 °C for Akt3, and 58 °C for G3PDH), and extension at 72 °C; and a final extension for 5 min at 72 °C. The DDBJ/EMBL/GenBank accession numbers for mouse Akt1, Akt2, Akt3, and G3PDH cDNA sequences are X6568, U2244, AF12414, and M32599, respectively.

**Results**

**Akt activity in oocytes is involved in meiotic resumption**

Inhibition of Akt activity using SH-6 during oocyte meiotic resumption was assessed using a light microscope with the Microscopy Relief Contrast System (Olympus, SZ). SH-6-treated oocytes exhibited GVBD (control, 98.3±1.7%; 20 μM, 95.4±2.3%; and 40 μM, 95.7±2.2%); however, progression to MI (control, 97.7±2.2%; 20 μM, 72.2±4.0%; and 40 μM, 27.8±4.0%) was inhibited by SH-6 in a dose-dependent manner (Figs. 1A, B). To address the effect of Akt inhibition on the nuclear status and microtubules, we performed an immunohistochemical analysis. As illustrated in Figs. 1C and D, SH-6 disturbed the formation of spindles at 10 h, although chromosomes appeared at 8 h. At 40 μM SH-6, the chromosomal alignment was abnormal. Surprisingly, lamin B, a key molecule of the nuclear lamina, was still located around the chromosomes at 10 h after the start of culture.
Thr308- and Ser473-phosphorylated Akt function individually and are both necessary to assemble the MII spindle

Ten hours after the start of culture, MI oocytes were exposed to a medium containing 20 or 40 μM SH-6 and cultured for 8 h. As illustrated in Fig. 2A, at 18 h after the start of culture, the morphological PB1 emission did not differ with or without SH-6 (control, 99.0±1.0%; 20 μM, 93.4±3.4%; and 40 μM, 93.3±1.7%). The results of the immunohistochemical analysis with a confocal scanning laser microscope revealed that no phosphorylated Akt was present in the SH-6-treated MII oocytes and shorter spindles in Akt-inhibited oocytes although the chromosomal alignment was normal (Figs. 2B–D). These results suggest that Akt participates in spindle formation in MII oocytes.

Akt becomes phosphorylated at two residues, Thr308 and Ser473, and both are required for the full activation of Akt (Alessi et al., 1996). We have previously shown that Ser473-phosphorylated Akt had a similar distribution to that of microtubules, while Thr308-phosphorylated Akt was present in PCM in MI and MII oocytes (Hoshino et al., 2004). The difference in the localization of phosphorylated Akt suggests that the role of each active form may be different. To address this issue, we injected an antibody for each phosphorylated Akt into MI oocytes. Either the Thr308- or Ser473-phosphorylated Akt antibody caused a shorter spindle to form in MII oocytes (Fig. 3). Although spindles were shorter and abnormal in oocytes injected with the Thr308-phosphorylated Akt antibody, Ser473-phosphorylated Akts were still present in microtubules. Furthermore, injection of the Ser473-phosphorylated Akt antibody also produced a shorter and abnormal spindle, while Thr308-phosphorylated Akt was located in PCM. These results suggest that both forms are necessary for assembling the MII spindle and that Thr308- and Ser473-phosphorylated Akts function individually.

Individual Thr308- and Ser473-phosphorylated Akt activities in MII oocytes are involved in fertilization to complete meiosis

During post-fertilization, Thr308-phosphorylated Akt was located at the center of the midbody at anaphase with less intensity as compared to that in the MII oocytes (Figs. 4A, a). This expression disappeared at telophase (Figs. 4B, b) and the pronuclear stage (Figs. 4C, c). In contrast, Ser473-phosphorylated Akt still had a similar distribution to microtubules at anaphase (Figs. 4D, d), whereas it was extruded with PB2 from the ooplasm (Figs. 4E, e). At the pronuclear stage, Ser473-phosphorylated Akt was not detected (Figs. 4F, f). These results suggest that Akt activity may be associated with fertilization.

To address this issue, we examined the in vitro fertilization with 20 μM SH-6 of in vivo ovulated MII oocytes. Against all expectations, pronuclear formation rate did not differ between

Fig. 1. SH-6, an inhibitor of Akt activity, suppresses the complete breakdown of the nuclear envelope followed by a disruption of the chromosomal alignment and spindle formation. Rates of GVBD (A) and MI (B) were assessed without staining using a light microscope with the Microscopy Relief Contrast System at 8 and 10 h, respectively, after the start of in vitro maturation. The percentage rate of GVBD (control, n=57; 20 μM, n=47; and 40 μM, n=47) was unaffected (A), while progression to MI (control, n=35; 20 μM, n=35; and 40 μM, n=35) at 10 h was inhibited by SH-6 in a dose-dependent manner (B). Values are the mean±S.E.M. of 3 replicates. Data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher’s protected least significant difference (PLSD). **Values with different superscripts are significantly different (P<0.05). Distributions of microtubules, chromosomes, and lamin B at 8 h (C) and 10 h (D) assessed by immunohistochemical analysis using a confocal scanning laser microscope. Green, red, and blue represent lamin B, chromosomes, and microtubules, respectively. Note the remains of lamin B with the disrupted chromosomes and spindle. Bar, 20 μm.
the control (93.3%) and SH-6 (87.2%). Although the fertilization rate was not affected by SH-6, Akt activity inhibition resulted in a shorter MII spindle. Therefore, the shorter spindle in MII oocytes may interrupt the process of fertilization. To address this hypothesis, MII oocytes treated with SH-6 in MI were fertilized in medium containing SH-6. As illustrated in Fig. 5A, neither the control nor the SH-6 treatment affected the penetration by sperm (control, 89.5±2.7%; 20 μM, 84.6±0.9%; and 40 μM, 84.0±1.4%), whereas PB2 emission was inhibited in a dose-dependent manner (control, 87.3±4.0%; 20 μM, 18.7±1.3%; and 40 μM, 0±0%). Immunohistochemical analysis of the eggs showed disorganized microtubules and chromosomal misalignment followed by a failure of PB2 emission (Fig. 5B).

In the present study, we demonstrated that Thr308- and Ser473-phosphorylated Akts function individually. Although the inhibition of either of the Akts resulted in a shorter spindle, the distribution of Thr308- and Ser473-phosphorylated Akts is completely different in MII oocytes. To determine whether or not Thr308- and Ser473-phosphorylated Akts have different functions in the fertilization of MII oocytes, we performed in
two active forms have different roles, i.e., Ser473-phosphorylated Akt activity is involved in PB2 emission while Thr308 regulates the organization of microtubules.

**High-level Akt expression during meiotic maturation disappeared during pre-implantation development**

Using immunohistochemical analysis, we have previously demonstrated that Akt is expressed during meiotic maturation

![Image](image_url)
In the present study, our results suggest that Akt disappears after fertilization. To address whether or not the Akt protein is re-expressed, we examined the expression of Akt protein and mRNA during oocyte meiotic maturation and embryonic development. By Western blot analysis, similarly high levels of phosphorylated and total Akts were detected from GV to MII during meiotic maturation (Fig. 6A). Akt has three isoforms (Akt1, Akt2, and Akt3) that are differentially expressed in a variety of tissues (Kandel et al., 2002). As illustrated in Fig. 6B, Akt1 and Akt3 mRNA were expressed whereas Akt2 mRNA was not detected. These results suggest that Akt1 and Akt3 are involved in spindle function and PB2 emission during meiotic maturation. In contrast to the oocytes, total Akt protein and mRNA in embryos were expressed at very low to undetectable levels at all stages of pre-implantation development (Figs. 6C–E). These results suggest that Akt function in the spindle is oocyte specific, to complete meiotic maturation through PB2 emission.

Discussion

We have previously demonstrated that exposure to LY294002, an inhibitor of PI3K activity, resulted in very low to undetectable levels of Thr308-phosphorylated Akt and an aberrant distribution of Ser473-phosphorylated Akt at MI in oocytes (Hoshino et al., 2004). In the present study, our results revealed that the inhibition of Akt induced incomplete GVBD followed by a failure of MI. These results suggest that complete GVBD is dependent on Akt acting via PI3K.

Full activation of Akt requires phosphorylation of both Thr308 and Ser473. The phosphorylation of Thr308, which is catalyzed by 3-phosphoinositide-dependent protein kinase-1 (PDK1), is in the activation loop (Kim et al., 2001). The identity of the putative Ser473 kinase (PDK2) remains controversial. Recently, it was demonstrated that a DNA-dependent protein kinase, which belongs to the PI3K superfamily, could be the putative Ser473 kinase (Feng et al., 2004). The phosphorylation of Ser473 in response to insulin or ionizing radiation is me-
Actin filament nucleator, has been identified and/or played roles in microtubule stabilization (Onishi et al., 2007). The microtubule requirement in insulin-stimulated GLUT4 redistribution is probably between PI3-kinase activation and the full activation of Akt/protein kinase B (PKB). This may occur at the level of PDK1 activation as Thr308 phosphorylation is significantly reduced in nocodazole-treated cells (Eyster et al., 2006). These reports demonstrate that the PI3K and Akt pathways play a role in the formation and stabilization of microtubules.

In our previous and present studies, results revealed that both the phosphorylated Akts participate in spindle function at MI oocytes as downstream effectors of the PI3K pathway. Thr308- and Ser473-phosphorylated Akts function individually and both are necessary for MI spindle assembly and for completion of fertilization during meiosis, that is, Ser473-phosphorylated Akt is involved in PB2 emission, whereas Thr308-phosphorylated Akt regulates the organization of microtubules.

Female meiotic divisions in higher organisms are asymmetric and lead to the formation of a large oocyte and small polar bodies. These asymmetric divisions are due to eccentric spindle positioning that, in mice, requires actin filaments. Recently, formin-2, a straight actin filament nucleator, has been proposed to control spindle positioning, chromosome segregation, as well as first polar body extrusion in mouse oocytes. Formin-2 controls first meiotic spindle migration to the cortex but not chromosome congression or segregation. Furthermore, the lack of first polar body extrusion in fmn2(−/−) oocytes is not due to a lack of cortical differentiation or central spindle formation but due to a defect in the late steps of cytokinesis (Dumont et al., 2007). The Akt pathway is known as one of the formin-2 signaling pathways (Favaro et al., 2003). These reports indicate the possibility that Akt is related to polar body emission in mouse meiosis.

Mice with a targeted disruption of Akt1 and/or Akt2 have been obtained, with Akt1 mutant mice displaying an increased neonatal lethality and a reduction in body weight of ~30% (Cho et al., 2001b; Yang et al., 2003). Moreover, loss of Akt1 leads to placental hypotrophy with impaired vascularization (Yang et al., 2003). In contrast, Akt2-deficient mice are born with the expected Mendelian ratio and exhibit a diabetes-like syndrome with an elevated fasting plasma glucose level, hepatic glucose output, and peripheral insulin resistance, and a compensatory increase of islet mass (Cho et al., 2001a). Compared with Akt1 mutant mice, Akt2-deficient mice are only mildly growth retarded (Cho et al., 2001a; Garofalo et al., 2003). However, mice lacking both isoforms die after birth, probably due to respiratory failure (Peng et al., 2003). Akt1−/−/Akt2−/− double mutant newborns display a severe reduction in body weight (~50%), prominent atrophy of the skin and skeletal muscle, impaired adipogenesis, and delayed ossification. In contrast to Akt1 and Akt2-deficient mice, Akt3−/− mice are viable and lack increased perinatal mortality, growth retardation, or altered glucose metabolism. However, loss of Akt3 profoundly affects postnatal brain growth (Easton et al., 2005; Tschopp et al., 2005). Ablation of a single copy of Akt3 in Akt1-deficient mice (Akt1−/−/Akt3+/−) led to a higher perinatal mortality as compared with Akt1 single mutant mice and the ablation of both Akt3 alleles in Akt1−/−/Akt3−/+ mice led to more pronounced dwarfism and intrauterine death of all Akt1−/−/Akt3−/+ double mutant animals (Tschopp et al., 2005). However, it cannot yet be confirmed whether or not the observed phenotypes are due to a combination of reduced activated Akt levels and the loss of isoform-specific functions. In contrast, oocytes at GV contain 4n chromosomes in heterozygous female mice, so that Akt-deficient embryos would lose Akt expression after zygotic gene activation. Therefore, the phenotype of Akt-deficient oocytes remains unclear.

In the present study, Akt1 and Akt3 mRNA were expressed whereas Akt2 was not detected in mouse oocytes during meiosis. Furthermore, antibodies for Akt detected endogenous Akt1, Akt2, and Akt3 proteins. Therefore, Akt1 and Akt3 are involved in spindle function and PB2 emission, although it remains unclear whether the observed incomplete meiosis in Akt-inhibited oocytes is due to a combination of reduced activated Akt levels. The different functions of Thr308- and Ser473-phosphorylated Akt could be due to different isoforms with different phosphorylated residues.

During post-fertilization, Thr308-phosphorylated Akt disappeared at anaphase II and Ser473-phosphorylated Akts were extruded with the PB2 from ooplasm. In contrast to meiosis in the oocytes, total Akt protein and mRNA in embryos were expressed at very low to undetectable levels at all stages of pre-implantation development. Akt-deficient mice exhibit no evidence of the loss of embryos during pre-implantation development. Indeed, Akt2-deficient mice are born with the expected Mendelian ratio (Cho et al., 2001a) and Akt3−/− mice are viable without increased perinatal mortality and growth retardation (Easton et al., 2005; Tschopp et al., 2005), whereas an Akt1-deficiency in embryos mostly results in neonatal lethality (Cho et al., 2001b). These results suggest that Akt is not essential for pre-implantation development after zygotic gene activation in mouse embryos. Riley et al. (2005) detected Akt at the plasma membrane throughout the pre-implantation development of embryos. Although our findings are not consistent with those of Riley et al. (2005), both our results and those of Riley et al. demonstrated very low to undetectable levels of Akt expression in the cytoplasm and spindle in embryos. These results suggest that the function of Akt in the spindle is oocyte specific, to complete meiotic maturation via PB2 emission.

The activation of Akt depends on the phosphorylation at Thr308 and Ser473. It was shown previously that the Thr308 residue is phosphorylated by PDK1 and that membrane localization is a necessary criterion for Ser473 phosphorylation (Scheid et al., 2002; Troussard et al., 2003). New results have shown that in Drosophila and human somatic cells, the targets of rapamycin kinase and its associated protein rictor are...
necessary for the phosphorylation at Ser473 (Sarbassov et al., 2005). The meiosis-specific downstream pathway of Akt remains unclear. In mouse oocytes, inhibition of glycogen synthase kinase-3 (GSK-3) had no significant influence on viability, morphology, or development to MII, whereas the inhibitor caused an abnormal spindle to form and a significantly increased incidence of abnormal homologue segregation during the first meiotic division (Wang et al., 2003). Akt phosphorylates the downstream kinase GSK-3. In somatic cells, it is known that the mammalian target of rapamycin (mTOR) is a downstream target of Akt (Majumder et al., 2004). The distribution of phosphorylated mTOR was similar to that of Ser473-phosphorylated Akt in mouse meiosis (unpublished). Therefore, the PI3K–Akt–GSK-3 pathway could be associated with an oocyte-specific function during meiosis. In addition, mTOR also could be functions around Akt in meiosis.

This manuscript provides evidence that Ser473-phosphorylated Akts are involved in PB2 emission while Thr308-phosphorylated Akts regulate the organization of microtubules for the completion of meiosis in mouse oocytes. Further study is underway to elucidate the mechanism of Thr308 and Ser473 phosphorylated Akts in mouse meiotic maturation.

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