[Frontiers in Bioscience 11, 2193-2202, September 1, 2006]

Degradation of securin in mouse and pig oocytes is dependent on ubiquitin-proteasome pathway and is required for proteolysis of the cohesion subunit, Rec8, at the metaphase-to-anaphase transition

Li-Jun Huo [1, 2], Zhi-Sheng Zhong [1, 3], Cheng-Guang Liang [1,2], Qiang Wang [1, 2], Shen Yin [1, 2], Jun-Shu Ai [1, 2], Ling-Zhu Yu [1, 2], Da-Yuan Chen [1], Heide Schatten [3], and Qing-Yuan Sun [1]

[1] State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China [2] Graduate School, Chinese Academy of Sciences, Beijing 100080, China, [3] Department of Veterinary Pathobiology, University of Missouri-Columbia, MO 65211

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1. ABSTRACT

Although securin/separase/cohesion pathway was reported to regulate chromosome segregation during meiotic metaphase-to-anaphase transition, little biochemical evidence was provided. We recently found that oocytes could not progress beyond meiotic metaphase when ubiquitin-proteasome pathway was inhibited, but the mechanisms remain unclear. In the present study, we investigated the quantity of securin and Rec8 protein and the localization of securin, a cohesion subunit, during oocyte meiosis providing data in support of the hypothesis that the effect of ubiquitin-proteasome pathway on metaphase-to-anaphase transition was mediated by regulating securin and Rec8 degradation in mouse and pig oocytes. In germinal vesicle-stage oocytes, immunostaining of securin was mainly localized in the germinal vesicle. Shortly after germinal vesicle breakdown, immunoreactive securin accumulated around the condensed chromosomes at prometaphase I. At metaphase I and metaphase II, when chromosomes were organized at the equatorial plate, immunoreactive securin was concentrated around the aligned chromosomes, putatively associated with the position of the metaphase spindle. The accumulation of securin could not be detected at anaphase I and anaphase II. In both mouse and pig oocytes, Western blot analysis showed that securin protein was low at germinal vesicle stage, reached the highest level at metaphase I, while decreased at anaphase I. Securin was increased again at metaphase II, while it was decreased at anaphase II. Rec8 protein was present in germinal vesicle-stage oocytes and remained until metaphase I, while it was decreased at anaphase I. Like securin, Rec8 was increased at metaphase II, while it was decreased again at anaphase II. The inhibition of the ubiquitin-proteasome pathway inhibited the decrease in securin and Rec8 at metaphase-to-anaphase transitions in both mouse and pig oocytes. Microinjection of securin antibody into MII-arrested oocytes leads to the degradation of Rec8. In conclusion, these results suggest that the proteolysis of securin is dependent on ubiquitin-proteasome pathway and is necessary for the degradation of Rec8 during meiotic metaphase-to-anaphase transitions in mouse and pig oocytes.

2. INTRODUCTION

Sexually reproducing organisms rely on a specialized meiotic cell cycle for the maintenance of diploidy in their progeny. The meiotic cell cycle consists of a single DNA replication phase followed by two chromosome segregation processes, thus producing haploid gametes. The diploid complement is then restored in the zygote, when the gametes from each parent are united with each other. In the first meiotic division, homologous chromosomes segregate away from each other, and in the second meiotic division, which resembles mitotic division, sister chromatids separate (1, 2). Chromosome segregation during mitosis and meiosis II is triggered by disjunction of sister chromatid cohesion at the metaphase to anaphase transition, which is mediated by the cohesion complex (3). In somatic cells, cohesion appears to be present along the entire length of the chromosome, between centromeres and along chromosome arms (4). Mitotic sister chromatid disjunction requires the loss of cohesion along the entire length of chromosomes, whereas homologous chromosome segregation at meiosis I only requires loss of cohesion along the chromosome arms but not the cohesion around the centromeres. Then, in anaphase II, sister chromatids separate by releasing centromere cohesion (5). All of these steps are essential for faithful transmission of chromosomes and thereby must be regulated precisely. However, almost all of these results were obtained on yeast, *Xenopus, Drosophila* or other somatic cells. The molecular mechanisms regulating the homologous chromosome/sister chromatid segregation at the metaphase-to-anaphase transition during oocyte meiosis are still unclear in mammals.

In somatic cells, a multi-subunit protein complex called cohesion holds sister chromatids together until the onset of anaphase. Cohesion contains four proteins, Smc1, Smc3, Scc1/Rad21 and Scc3 (6, 7). The complex shows considerable evolutionary conservation from yeast to vertebrates, although in the latter somatic cells express two Scc3 homologues, STAG1 and STAG2 (8, 9). In all organisms investigated to date, meiosis-specific variants of somatic cohesion complex proteins have been identified. STAG3, SMC1b and Rec8 are thus meiosis-specific homologues of STAG1/ 2, SMC1a and Scc1/RAD21, respectively(10-12). Rec8 protein has been implicated in homologous chromosome/sister chromatid cohesion in meiosis I and II of yeast (13-16), worm (17), rat (18) mouse (19), and human(20)**,** whose cleavage mediated by separase triggered the chromosome/chromatid segregation at the metaphase to anaphase transition. Although the expression or roles of meiosis-specific variants of cohesion have been described in the male mouse(18, 19, 21-23), little is known about the expression patterns and regulatory mechanisms of Rec8 during oocyte meiosis in mammals, except that the cohesion component dynamics or mRNA expression have been described during meiotic prophase I in mouse and human oocytes(9, 24).

Separase is a thiol protease, which destroys chromosome/chromatid cohesion by cleaving cohesion's kleisin subunit Scc1 (5, 20, 25). Separase is kept inhibited for most of the cell cycle through its association with an inhibitory chaperone called securin (Pds1 in yeast).. Separase is activated at the metaphase to anaphase transition by the sudden Fizzy/Cdc20 and anaphase-promoting complex/cyclosome (APC/C)-dependent destruction of securin(26-33). It has been shown that the degradation of securin through ubiquitin-proteasome pathway (UPP) (APC or its activator protein Cdc20) is necessary for the onset of metaphase I to anaphase I transition in yeast and*Drosophila* (30, 34). Recently, securin and separase have also been suggested to play important roles in homologue disjunction during the first meiosis of mouse oocytes (35, 36). However, the dynamics of securin during oocyte meiosis in mammals is not yet known. It is necessary to clarify whether securin is decreased during the metaphase-to-anaphase transition, and if so, how the degradation of securin is mediated.

It has been known that ubiquitin-proteasome pathway (UPP) regulates the onset of the metaphase-to-anaphase transition in both mitosis and meiosis(37, 38). Studies by us and others also have shown that UPP inhibitor could arrest the oocyte meiotic maturation at metaphase I and prevent the activation of MII oocytes (arresting the oocyte at metaphase II)(39, 40). In cells including mammalian oocytes, cyclin B is degraded through UPP, which leads to the inactivation of maturation or M-phase promoting factor (MPF), and finally triggers the onset of anaphase(35, 39, 41, 42). It was also suggested that the degradation of securin mediated by UPP resulted in the activation of separase, and the separase leads to the cleavage of cohesion subunits, finally, the cleavage of cohesion subunits is necessary for the metaphase-to-anaphase transition in yeast and *Drosophila* (28, 38, 43). Despite this recent progress in our understanding of the biochemical basis of cell cycle regulation, however, to our knowledge, little is known about the molecular control of meiotic metaphase-to-anaphase transition in mammalian oocytes. Particularly, only limited information exists about the relationship among these pathways, such as the degradation mediated by UPP, the interaction of securin and separase, and the proteolysis of cohesion subunits at the metaphase to anaphase transition(44-46).

In this study, by using large quantities of oocytes for biochemical analysis, we found that the quantity of both securin and Rec8 protein was decreased at the metaphase I/anaphase I transition and metaphase II/anaphase II transition in mouse and pig oocytes, which is consistent with the reports in yeast and *Drosophila* (30, 34, 47-50). Furthermore, the inhibition of UPP could prevent securin/cohesion degradation and meiotic metaphase-to-anaphase transition. Microinjection of securin antibody into MII-arrested oocytes lead to the degradation of Rec8 in mouse oocytes. Based on our results and other reports, we propose that the degradation of securin and rec8 at meiotic metaphase I/anaphase I transition and metaphase II/anaphase II transition depends on UPP. The proteolysis of securin dependent of UPP is necessary for the activation of separase, which leads to the degradation of cohesion (rec8) and finally the segregation of homologous chromosomes and sister chromatids.

3. MATERIALS AND METHODS

3.1. Chemicals and Solutions

Monoclonal mouse anti-human securin antibody (ab3305) was purchased from Abcam Ltd (EMD Biosciences, Inc. San Diego, CA), and polyclonal goat anti-human rec8 antibody (sc-15152) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The inhibitor of ubiquitin-proteasome pathway, MG-132 (*Z*-Leu-Leu-Leu-CHO), purchased from Calbiochem (La Jolla, CA), was diluted as 10mM stock solution in dimethyl sulfoxide (DMSO) and stored at −20 °C in a dark box. The stock solution was diluted in M2 or M199 medium just prior to use. All other chemicals or components of media were embryo culture or cell culture grade and were obtained from Sigma (St. Louis, MO) unless noted otherwise.

3.2. Mouse Oocyte Collection and Culture

Kunming mice, a native breed widely used in biological research in China, were used for oocyte and zygote collection. Animal care and handling were conducted in accordance with policies promulgated by the ethical committee of the Institute of Zoology, Chinese Academy of Sciences. Fully grown germinal vesicle-intact oocytes were obtained as previously described by Tong (51) and maintained in M2 medium supplemented with 60 μg/ml penicillin and 50 μg/ml streptomycin, and cultured in M2 medium. All cultures were carried out at 37 ºC in a humidified atmosphere of 5% CO_2,. At 2h after culture, the oocytes that had undergone GVBD were further cultured for 10h, and the oocytes were collected at different stages for confocal microscopy or Western blot. Meanwhile, at 2h after culture, the oocytes that had undergone GVBD were further cultured in M2 medium containing 10 μM MG-132 for 10h and then collected for Western blot or washed thoroughly and cultured in M2 medium for additional 2h and then collected for Western blot.

For the collection of metaphase II-arrested eggs, females were superovulated by intraperitoneal injection with 10 IU of pregnant mares' serum gonadotropin (PMSG) followed 46-48 h later by 10 IU of human chorionic gonadotropin (hCG). Mice were sacrificed and oviducts were removed at 14-16 h after hCG injection. Using a pair of fine forceps to tear the oviducts, cumulus masses were collected in M2 medium. To remove the cumulus cells, eggs were briefly exposed to 300 IU/ml hyaluronidase followed by three washes in M2 medium. The denuded MII-arrested eggs were collected and used for parthenogenetic activation, or for confocal microscopy, Western blot or microinjection of securin antibody.

3.3. Pig Oocyte Collection and Culture

Ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 1 h. Oocytes were aspirated from antral follicles (2~6 mm in diameter) with an 18-gauge needle fixed to a 20-ml disposable syringe. After three washes with maturation medium (see below), oocytes with compact cumulus and evenly granulated ooplasm were selected for maturation culture. The medium used for maturation culture was TCM-199 (Gibco, Grand Island, NY) supplemented with 75 μg/ml potassium penicillin G, 50μg/ml streptomycin sulphate, 0.57 mM L-cysteine, 0.5 μg/ml FSH, 0.5 μg/ml LH and 10 ng/ml epidermal growth factor. A group of 25 oocytes was cultured in a 100-μl drop of maturation medium for 44 h at 39^{o}C in an atmosphere of 5% CO_2 and saturated humidity. The oocytes at different stages were collected for confocal microscopy or Western blot. For inhibitor treatment, the oocytes at 24h after maturation culture were cultured in TCM-199 medium containing 10 μM MG-132 for 20h and then collected for Western blot or washed thoroughly and cultured in TCM-199 medium for additional 2h and then collected for Western blot.

After maturation culture, oocytes were freed of cumulus cells by treatment with 300 IU/ml hyaluronidase (Sigma) and repeated pipetting. The denuded oocytes were then washed twice in TCM-199 and used for confocal microscopy, Western blot or parthenogenetic activation.

3.4. Parthenogenetic Activation of Oocytes

Parthenogenetic activation of mouse oocytes was performed as previously reported by us (51). The denuded MII-arrested eggs were treated with 10 μM A23187 for 5 min, and then incubated in M16 medium containing 10 μg/ml CHX. The activated eggs were collected at 1.5h for confocal microscopy or Western blot.

In vitro matured pig oocytes were induced to undergo parthenogenetic activation by electrical pulse stimulation, a method essentially the same as described by Fan et al. (52). Briefly, after washing three times in the electroporation medium (0.28 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.01% (w/v) BSA), cumulus-free oocytes were placed in a fusion chamber. An 80 μs pulse at 120 V/mm DC was applied to oocytes. The oocytes were then washed 3 times and cultured in NCSU-23 medium containing 0.4% BSA. The oocytes at 2h after culture were collected for confocal microscopy or Western blot.

For the inhibitor treatment, the *in vitro* matured pig eggs or MII-arrested mouse eggs were first cultured for 1h in TCM-199 (for pig oocytes) or M2 medium (for mouse oocytes) containing 10 μM MG-132, then used for parthenogenetic activation. 10 μM MG-132 (final concentration) was always included in the medium during the process of parthenogenetic activation.

3.5. Confocal Microscopy of Oocytes

After removal of zona pellucida (ZP) in acidified M2 medium (pH 2.5), mouse oocytes at the desired stage were fixed in 4% paraformaldehyde in PBS for 30 min and permeabilized for 30 min in the incubation buffer (0.5% Triton X-100 in 20mM Hepes, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose, 0.02% NaN_3), then the oocytes were washed in PBS with 0.1% Tween 20 for three times and then incubated with monoclonal mouse anti-human securin antibody diluted 1:50 for 1 h. The oocytes were rinsed three times and incubated for 1 h with 1:100 FITC-conjugated goat anti-mouse IgG, followed by staining with 10 μg/ml propidium iodide. Finally, the oocytes or fertilized eggs were placed on a glass slide, mounted in 1,4-diazabicyclo(2.2.2)octane hydrochloride-containing

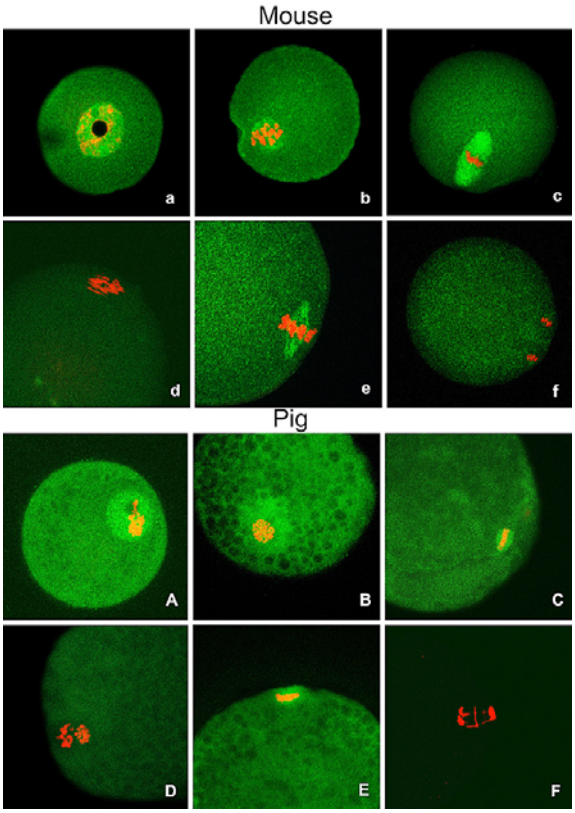

Figure 1. Subcellular localization of securin during oocyte meiotic maturation and activation in mouse and pig. Green, securin; Red, chromatin. The lower case and capital letters represent the results obtained in mouse and pig, respectively. The immunostaining of securin was mainly localized to the germinal vesicle at GV-stage (a and A). At prometaphase I, the immunostaining of securin was concentrated at the periphery of condensed chromosomes (b and B). At metaphase I (c and C) and metaphase II (e and E), the immunostaining of securin was accumulated around the aligned chromosomes, putatively the position of the metaphase spindle. At anaphase I (d and D) and anaphase II (f and F), immunolocalization of securin was absent.

medium, and covered with a cover glass. The samples were examined using a TCS-4D laser scanning confocal microscope (Leica Microsystems, Bensheim, Germany). Pig oocytes were permeabilized with 1% Triton X-100 in PBS overnight at 37 ^{o}C after fixation as described above, and the other protocols were the same as described for the mouse.

The configuration of spindles in mouse oocytes was determined by incubating the oocytes in 1: 50 FITC-conjugated anti-α tubulin antibody for 1 h after fixation and permeabilization as described above.

3.6. Microinjection of Securin Antibody

Monoclonal mouse anti-human securin antibody (100 μg/ml in PBS) was injected into the MII-arrested mouse oocytes as described by Tong et al (51). An Eppendorff microinjector was used in this experiment. All microinjections were performed by using a beveled micropipette to minimize damage and were completed within 30 min. A microinjection volume of about 7 pl per oocyte was used in all the experiments. Each experiment consisted of three separate and replicate groups and approximately 200 oocytes were injected in each group. The same amount of mouse IgG was injected into the oocytes as the negative control. After microinjection of securin antibody, the oocytes were cultured in M2 medium for 6h and then collected for Western blot or confocal microscopy.

3.7. Western Blot Analysis

For detection of securin, oocytes were collected in SDS sample buffer and heated to 100 ^{o}C for 4 min. Equal numbers of oocytes (100 oocytes) were loaded in all groups. After cooling on ice and centrifuging at 12,000 g for 3 min, samples were frozen at –20 ^{o}C until use. The total proteins were separated by SDS-PAGE with a 5% stacking gel and a 15% separating gel for 30 min at 90 V and 2.5 h at 120 V, respectively, and then electrophoretically transferred onto PVDF membrane for 2.5 h, 200 mA, at 4 ^{o}C. Then the membrane was blocked for 2h at room temperature in TBST buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.4) containing 5% low-fat milk and further incubated overnight at 4 ^{o}C in TBST with 1:300 monoclonal mouse anti-human securin antibody. After 3 washes of 10 min each in TBST, the membranes were incubated for 1 h at 37 ^{o}C with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG diluted 1:1000 in TBST. The membranes were washed three times in TBST and then processed using the enhanced chemiluminescence plus (ECL plus) detection system (Amersham Pharmacia Biotech, UK). All experiments were repeated at least three times.

For detection of rec8, proteins from 200 oocytes were extracted, separated, and transferred onto the nitrocellulose membrane as mentioned above. The membranes were blocked with 5% low-fat milk in TBST for 1 h at 37 ^{o}C, and then incubated overnight with polyclonal goat anti-human rec8 antibody diluted 1:300 at 4 ^{o}C. The procedure of second antibody binding, washing, and ECL processing was the same as that for securin detection.

4. RESULTS

4.1. Subcellular localization of securin during oocyte meiotic maturation and activation in mouse and pig

The distribution of securin during oocyte meiotic maturation and activation in the mouse and pig is shown in Figure 1. The localization of securin in mouse and pig oocytes was similar at the same stage of meiosis. At GV-stage, securin mainly accumulated in the germinal vesicle (Figure 1a and A). Shortly after GVBD, securin was concentrated at the periphery of condensed chromosomes at prometaphase (Figure 1b and B). At metaphase I (Figure 1c and C) and metaphae II (Figure 1e and E), the staining of securin was observed around the aligned chromosomes, putatively the position of the metaphase spindle. At anaphase I (Figure 1d and D) and anaphase II (Figure 1f and F), securin was not detectable.

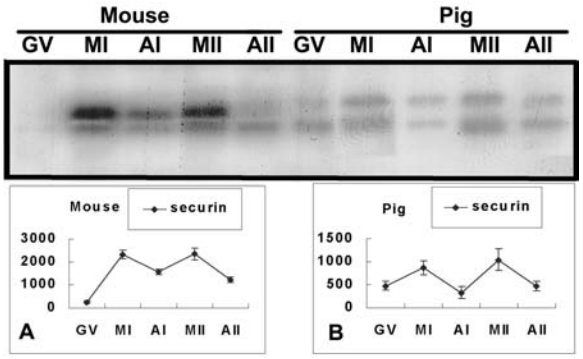

Figure 2. Quantity of securin protein during oocyte meiotic maturation and activation in mouse and pig. The oocytes at different stages were collected for Western blot. For mouse, oocytes were collected at GV stage (GV 0h), metaphase I (GVBD+6h I), anaphase I (GVBD+7.5h), metaphase II and anaphase II (1.5hafter parthenogenetic activation). For pig, oocytes were collected at 0h (GV), 30h (metaphase I), 32h (anaphase I), 44h (metaphase II) after maturation culture and 2h (anaphase II) after parthenogenetic activation. Overall, the dynamics of securin protein between mouse oocytes and pig oocytes was similar. At GV-stage (panels 1 and 6), anaphase I (panels 3 and 8) and anaphase IIs (panels 5 and 10), the level of securin was low, while securin was abundant at metaphase I (panels 2 and 7) and metaphase II (panels 4 and 9). Quantification of securin was expressed as A and B for mouse and pig, respectively. Values shown are the mean ± SEM from three independent experiments.

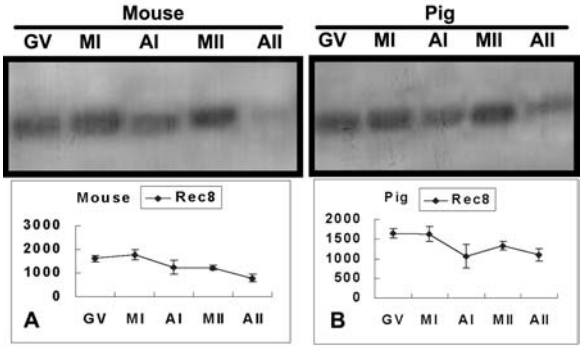

Figure 3. Quantity of Rec8 protein during oocyte meiotic maturation and activation in mouse and pig as revealed by Western blot. The oocytes collected were the same as for detection of securin. Overall, the dynamics of Rec8 protein between mouse and pig was similar. At GV-stage (panels 1 and 6), metaphase I (panels 2 and 7), and metaphase II (panels 4 and 9), Rec8 was abundant, but its quantity was decreased at anaphase I (panels 3 and 8) and at anaphase II (panels 5 and 10). Quantification of Rec8 was expressed as A and B for mouse and pig, respectively. Values shown are the mean ± SEM from three independent experiments.

4.2. Quantity of securin protein during oocyte meiotic maturation and activation in mouse and pig

As shown in Figure 2, the quantity of securin protein was detected by Western blot during oocyte meiotic maturation and activation in mouse and pig. For the mouse, oocytes at 8h and 9.5h after culture were collected for detection of securin at metaphase I and anaphase I, respectively. For the pig, oocytes at 30h, 32h and 44h after maturation culture were collected for detection of securin at metaphase I, anaphase I, and metaphase II, respectively. Overall, the quantity of securin protein in mouse oocytes was similar to that in pig oocytes. At GV-stage, the quantity of securin protein was very low (Figure 2, panels 1 and 6), but increased at metaphase I (Figure 2, panels 2 and 7). At anaphase I (Figure 2, panels 3 and 8), the level of securin was decreased. At metaphase II (Figure 2, panels 4 and 9), the level of securin was increased, while at anaphase II (Figure 2, panels 5 and 10), the level of securin was decreased again.

4.3. Quantity of Rec8 protein during oocyte meiotic maturation and activation in mouse and pig

As shown in Figure 3, the quantity of Rec8 protein was detected by Western blot during oocyte meiotic maturation and activation in mouse and pig. The dynamics of Rec8 protein in mouse oocytes was consistent with that in pig oocytes. A high level of Rec8 could be detected from GV stage (Figure 3, panels 1 and 6) to metaphase I stage (Figure 3, panels 2 and 7), it decreased slightly at anaphase I (Figure 3, panels 3 and 8), increased at metaphase II (Figure 3, panels 4 and 9), and decreased again at anaphase II (Figure 3, panels 5 and 10).

4.4. Inhibition of ubiquitin-proteasome pathway prevents the degradation of securin at the metaphase-to-anaphase transition in mouse and pig oocytes

For the first metaphase-anaphase transition, the inhibitor of the ubiquitin-proteasome pathway, MG-132 (10μM final concentration), was added to the culture medium after GVBD at 2h in mouse or added at 24h after culture in pig, then the oocytes were cultured for an additional 10h for mouse or 20h for pig. At the end of culture, the oocytes were collected for Western blot or washed thoroughly and then cultured in inhibitor-free medium for an additional 2h, then the oocytes were collected for Western blot. As shown in Figure 4, the level of securin was high in oocytes cultured in inhibitor-containing medium (Figure 4, panel 1) but was very low in oocytes cultured in inhibitor-free medium for an additional 2h (Figure 4, panel 2). For the second metaphase-anaphase transition, the MII-stage oocytes were first treated with 10μM MG-132 (final concentration) for 1h and then used for parthenogenetic activation. 10μM MG-132 was always included in the medium during the process of parthenogenetic activation. As a control, the oocytes were incubated in DMSO-containing medium (0.1%) for 1h and then performed for parthenogenetic activation and DMSO (0.1%) was always included in the medium. Our results showed that the level of securin was abundant (Figure 4, panel 3) in oocytes treated with MG-132 but was significantly decreased in control oocytes (Figure 4, panel 4).

4.5. Inhibition of ubiquitin-proteasome pathway prevents the degradation of Rec8 during the metaphase-anaphase transition in mouse and pig oocytes

For Rec8, the treatment of oocytes was the same as that mentioned above. As shown in Figure 5, for the first

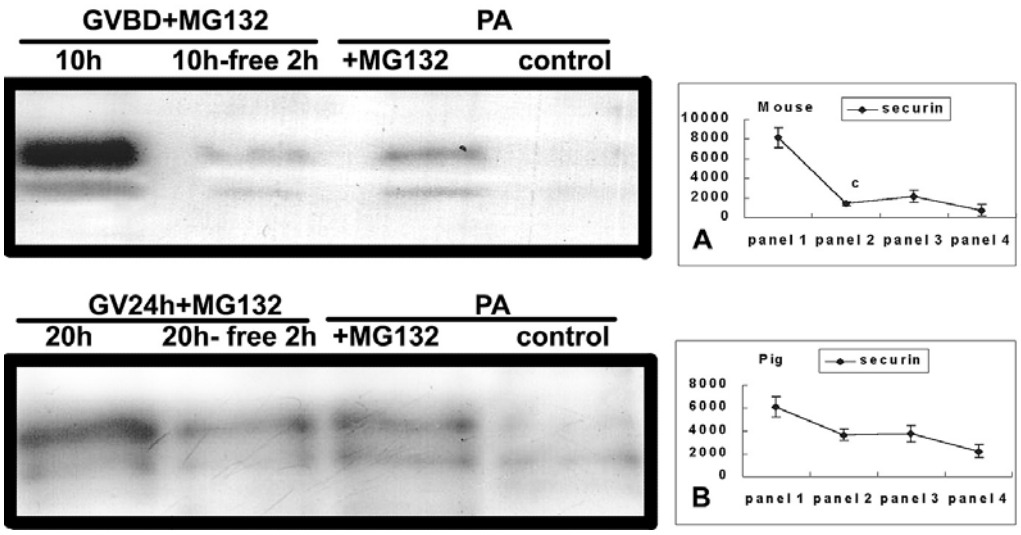

Figure 4. Inhibiton of ubiquitin-proteasome pathway prevents the degradation of securin during the metaphase-to-anaphase transition in mouse and pig oocytes. For the first metaphase-to-anaphase transition, the inhibitor of ubiquitin-proteasome pathway, MG-132 (10μM final concentration), was added into M2 medium after oocyte GVBD in mouse or added into M199 medium at 24 h after oocyte culture in pig, then the oocytes were cultured for an additional 10h for mouse or 20h for pig. At the end of culture, the oocytes were collected for Western blot or washed thoroughly and then cultured in inhibitor-free medium for an additional 2h, then the oocytes were collected for Western blot. Securin was abundant in oocytes cultured in inhibitor-containing medium (panel 1) but was very low in oocytes cultured in inhibitor-free medium for an additional 2h (panel 2). For the second metaphase-to-anaphase transition, the MII-stage oocytes were first treated with 10μM MG-132 for 1h and then used for parthenogenetic activation. 10 μM MG-132 was always included in the medium during the process of parthenogenetic activation. As a control, the oocytes were incubated in M2 medium containing DMSO (0.1% as a final concentration) for 1h and then used for parthenogenetic activation. Securin was abundant (panel 3) in oocytes treated with 10μM MG-132 but its quantity was significantly decreased in control oocytes (panel 4). Quantification of securin was expressed as A and B for mouse and pig, respectively. Values shown are the mean ± SEM from three independent experiments.

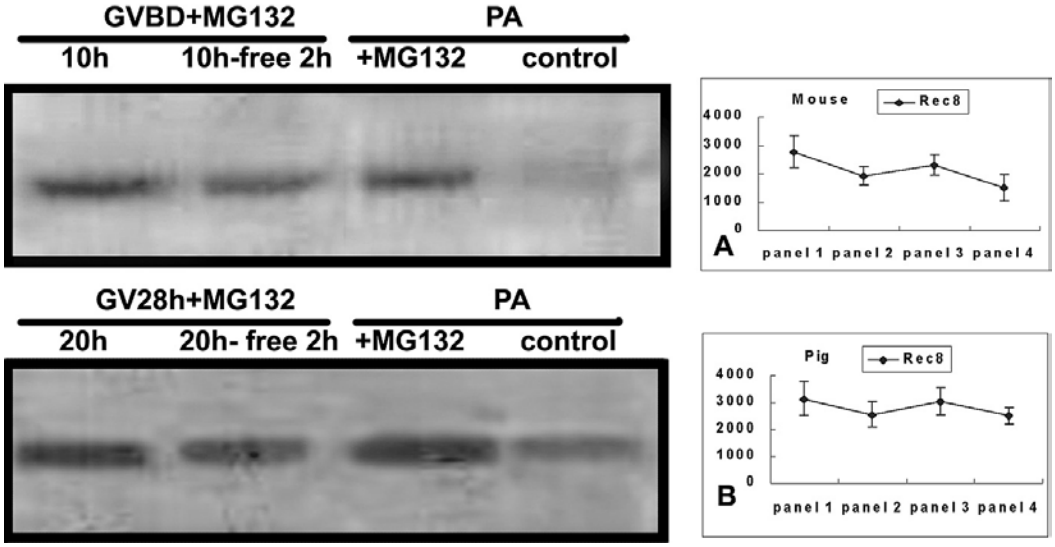

Figure 5. Inhibition of ubiquitin-proteasome pathway prevents the degradation of rec8 during the metaphase-to-anaphase transition in mouse and pig oocytes. For detection of rec8, the procedure for treatment and collection of oocytes was the same as that for detection of securin. For the first metaphase-to-anaphase transition, Rec8 was abundant both in oocytes cultured in inhibitor-containing medium (panel 1) and decreased in oocytes cultured in inhibitor-free medium for an additional 2h (panel 2). For the second metaphase-to-anaphase transition, Rec8 was abundant (panel 3) in oocytes treated with 10μM MG-132 but was significantly decreased in control oocytes (panel 4). Quantification of Rec8 was expressed as A and B for mouse and pig, respectively. Values shown are the mean ± SEM from three independent experiments.

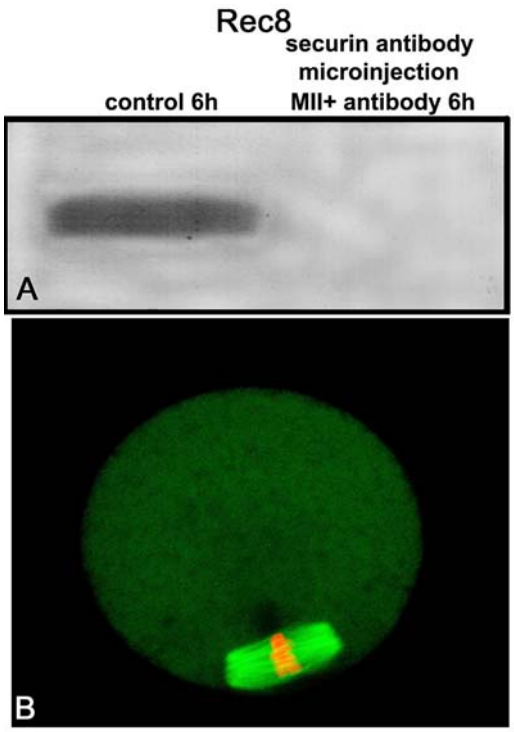

Figure 6. Microinjection of securin antibody at metaphase II promotes the degradation of rec8 during oocyte parthenogenetic activation in mouse. After microinjection of securin antibody, the injected oocytes (at metaphase II) were cultured for 6h in M16 medium and then collected for Western blot or confocal microscopy. Rec8 was detected in control oocytes (panel 1) but was absent in oocytes microinjected with securin antibody (panel 2). The results obtained by immunostaining of α-tubulin revealed that the oocytes at 6h after microinjection of securin antibody were still arrested at metaphase II (Figure 6B).

metaphase-to-anaphase transition, the level of Rec8 was high in oocytes cultured in inhibitor-containing medium (Figure 5, panel 1), while it was decreased in oocytes cultured in inhibitor-free medium for an additional 2h (Figure 5, panel 2). For the second metaphase-to-anaphase transition, our results showed that the level of Rec8 was high (Figure 5, panel 3) in oocytes treated with MG-132, but was significantly decreased in control oocytes (Figure 5, panel 4).

4.6. Microinjection of securin antibody at metaphase II promotes the degradation of rec8 in mouse oocytes

After microinjection of securin antibody, the injected MII-arrested mouse oocytes were cultured for 6h in M2 medium and then collected for Western blot or confocal microscopy. As shown in Figure 6, the Rec8 could be detected in control oocytes (Figure 6A, panel 1), but was absent in oocytes microinjected of securin antibody (Figure 6A, panel 2). However, to our surprise the results obtained by immunostaining of α-tubulin revealed that the oocytes at 6h after microinjection of securin antibody were still arrested at metaphase II (Figure 6B).

5. DISCUSSION

In our study, we detected the dynamics of securin and Rec8 protein during oocyte meiosis in mouse and pig by Western blot, and the results showed that the quantity of securin or rec8 protein was decreased at the metaphase I/anaphase I transition and metaphaseII/ anaphase II transition. Securin was accumulated at the spindle region at metaphase I/II but disappeared at anaphase I/II. The inhibitor of UPP could prevent the degradation of securin and Rec8 at the metaphase I/anaphase I transition and metaphaseII/ anaphase II transition. Meanwhile, the quantity of Rec8 protein could not be detected at 6h after MII-arrested oocytes were microinjected with securin antibody. Based on our results and previous reports, we propose that degradation of securin and Rec8 at the metaphase-to-anaphase transition was mediated by the UPP pathway in mouse and pig oocytes. The proteolysis of securin may lead to the activation of separase that cleaves the cohesion subunit, Rec8, which is necessary for the meiotic metaphase-to-anaphase transition.

The level of securin was low at the GV stage, increased at metaphase I, significantly decreased at anaphase I, re-increased until metaphase II and significantly decreased again at anaphase II in mouse and pig oocytes. The dynamics of securin protein isconsistent with its reported roles during mitosis and meiosis in yeast, *Drosophila* and *Xenopus*, that is securin maintains chromosome pairing and its degradation at the onset of anaphase promotes the transition from metaphase to anaphase by inhibiting separase activity(30, 34, 47-50). Recently, it has been shown by immunofluorescent staining that securin−GFP gradually accumulated for 7−9 h after GVBD, declined over the next 2−3 h, then increased again. Polar body formation occurred at the end of the decline phase(35). Our findings for the first time provide biochemical evidence showing the important roles of securin in meiotic metaphase-to-anaphase transition in mammalian oocytes. Securin proteins were localized at the spindle but not the chromosomes in human HeLa cells(53). Our confocal results also showed that securin was accumulated around the aligned chromosomes, the position of metaphase I/II spindle but disappeared at anaphase I/II, providing further support for the role of securin in meiotic metaphase maintenance.

The degradation of securin is mediated by UPP (APC/Cdc20) in yeast and *Drosophila* or HeLa cells(53, 54). Our study directly shows that UPP inhibitor, MG-132, prevented the decrease of securin at the metaphase I/anaphase I transition and metaphase II/anapahse II transition in mouse and pig oocytes. Thus, the degradation of securin that is required for homologous chromosome separation during the first meiosis and sister chromatid separation during the second meiosis is probably mediated by UPP pathway. However, in *X. laevis* oocytes, reduction of APC/C-mediated proteolysis by several means (treatment with the proteasome inhibitor MG132, microinjection of antibodies against the APC/C activator fizzy, depletion of fizzy by antisense injection, microinjection of antibodies against the APC/C core

subunit Cdc27, injection of the APC/C inhibitor Mad2, and injection of undegradable securin) has no effect on the metaphase-to-anaphase transition in meiosis I, and oocytes are able to segregate their homologous chromosomes normally without degradation of cyclin B and securin, whereas reduction of APC/C-mediated proteolysis in meiosis II prevents the metaphase-to-anaphase transition (55, 56).

The dynamics of Rec8 protein was similar to that of securin during oocyte meiosis in mouse and pig, with only little variation. Rec8 was abundantly expressed at GV stage and maintained until metaphase I, slightly declined at anaphase I, re-increased at metaphase II, and then significantly declined at anaphase II. The decline of Rec8 at the metaphase I/anaphase I transition and metaphaseII/ anaphase II transition is consistent with that found in yeast, *Drosophila, S. cerevisiae, C. elegans,* mouse meiotic cells. Resolution of chiasmata depends on the cleavage of a meiosis-specific cohesion, Rec8, in meiosis (15, 17, 57). At the metaphase I-to-anaphase I transition, Rec8 is cleaved by separase along chromosome arms but is resistant to proteolytic cleavage in the vicinity of centromeres in yeast and mammalian meiosis(14, 19, 58, 59). Rec8 in the centromeric region is further cleaved by separase at the metaphase II-to-anaphase II transition (4, 14, 15, 19, 58, 60). For the slight decline of rec8 at metaphase I-to-anaphase I transition and significant decline at metaphse II-anaphase II transition, we propose two possible interpretations. First, because of the asynchronous progression of oocyte meiosis in mouse, there were about 30% at anaphase I at GV 9.5h after culture, which may account for why there was only a slight decline of rec8 at anaphase I, but about 85% oocytes entered anaphase after parthenogenetic activation, which may be the reason for the significant decrease of rec8 at anaphase II. Second, the cleavage of rec8 by separase at metaphase I-to-anaphase I transition takes place along chromosome arms but not around the centromeres, the latter will be further cleaved at meiosis II (19, 58, 59).

Our results showed that UPP inhibitor, MG-132, prevented the decline of Rec8 at the metaphase I/anaphase I transition and metaphase II/ anaphase II transition in mouse and pig. Based on these results and previous reports (5, 36, 61), we propose that UPP inhibitor prevents the degradation of securin at metaphase I/anaphase transition and metaphaseII /anaphase II transition in mammals, which inhibits the activation of separase, and thus also prevents the proteolysis of Rec8 by separase. Upon the onset of anaphase I/II, securin was degradated by UPP, followed by separase activation and cleavage of cohesion subunits (such as Rec8), then the homologous chromosomes/sister chromatids separated. Our results also showed that the level of Rec8 significantly decreased in oocytes microinjected with securin antibody, compared with that in control oocytes, which also supports our suggestion that the degradation of securin is necessary for the proteolysis of rec8 at meiotic metaphase-to-anaphase transition in mammalian oocytes. However, oocytes at 6h after microinjection with securin antibody were still arrested at metaphase II, as confirmed by an intact spindle with chromatids at the spindle equator detected by α-tubulin antibody and PI fluorescence staining (Figure 6B). Others' results also showed that despite rapid securin degradation induced by sperm penetration, sister chromatids remained attached in the presence of a D-box mutant of cyclin B1 (Delta 90 cyclin B1). This was a direct consequence of MPF activity because separation was induced following application of the MPF inhibitor roscovitine (62). Similar observations regarding the ability of MPF to prevent sister chromatid separation have recently been made in *Xenopus* egg extracts, mouse oocytes and in HeLa cells (50, 62). Overall, these results suggest that the proteolysis of both securin and Rec8 may be necessary but not sufficient for the separation of sister chromatids at the metaphase II to anaphase II transition. The degradation of cyclin B1, which leads to the inactivition of MPF, is also necessary for the separation of homologous chromosome/sister chromatid disjunction in mouse oocytes (35, 62). We propose that the degradation of cyclin B1 mediated by UPP, acting as an upstream signal, leads to the inactivation of MPF, which may be necessary for the activation of separase; meanwhile, the proteolysis of securin mediated by UPP is also essential for the activation of separase, and then the degradation of cohesion subunits (Rec8), both of which are necessary for the separation of homologous chromosomes/sister chromatids at the metaphase-to-anaphase transition in mammalian oocytes. The failure of any of these processes can result in aneuploidy or in failure of meiotic segregation. We cannot exclude other regulatory processes necessary for modulating the separation of chromosomes/sister chromatids at the metaphase-to-anaphase transition due to the complexity of molecular processes during oocyte meiosis.

6. ACKNOWLEDGEMENT

This work was supported by National Natural Science Foundation of China (No. 30225010, 30170358, 30570944) and Special Funds for Major State Basic Research Project (973) of China (No. G1999055902).

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Key Words: Securin, Cohesion, Ubiquitin-Proteasome Pathway, Meiosis Ovum, Egg

Send correspondence to: Dr Qing-Yuan Sun, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, #25 Beisihuan Xilu, Beijing 100080, China, Tel: 8610-8262-7593, Fax: 8610-6256-5689; E-mail: sunqy1@yahoo.com

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