Two p90 Ribosomal S6 Kinase Isoforms Are Involved in the Regulation of Mitotic and Meiotic Arrest in Artemia

Received for publication, February 3, 2014, and in revised form, April 16, 2014 Published, JBC Papers in Press, April 22, 2014, DOI 10.1074/jbc.M114.553370

Ru-Bing Duan, Li Zhang, Dian-Fu Chen, Fan Yang, Jin-Shu Yang, and Wei-Jun Yang

From the Key Laboratory of Conservation Biology for Endangered Wildlife of the Ministry of Education and College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, China

Background: Ribosomal S6 kinase (RSK) plays important roles in meiosis and mitosis.

Results: Ar-Rsk1 promoted cdc2 phosphorylation and thereby meiotic arrest, whereas Ar-Rsk2 knockdown resulted in mitotic arrest.

Conclusion: Ar-Rsk1, which lacks an ERK-docking motif, controls meiotic arrest, whereas Ar-Rsk2, which has an ERK-docking motif, controls mitotic arrest.

Significance: The data provide insight into the functions of RSK isoforms.

There are multiple isoforms of p90 ribosomal S6 kinase (RSK), which regulate diverse cellular functions such as cell growth, proliferation, maturation, and motility. However, the relationship between the structures and functions of RSK isoforms remains undetermined. Artemia is a useful model in which to study cell cycle arrest because these animals undergo prolonged diapauses, a state of obligate dormancy. A novel RSK isoform was identified in Artemia, which was termed Ar-Rsk2. This isoform was compared with an RSK isoform that we previously identified in Artemia, termed Ar-Rsk1. Ar-Rsk2 has an ERK-docking motif, whereas Ar-Rsk1 does not. Western blot analysis revealed that Ar-Rsk1 was activated by phosphorylation, which blocked meiosis in oocytes. Knockdown of Ar-Rsk1 reduced the level of phosphorylated cdc2 and thereby suppressed cytostatic factor activity. This indicates that Ar-Rsk1 regulates the cytostatic factor in meiosis. Expression of Ar-Rsk2 was down-regulated in Artemia cysts in which mitosis was arrested. Knockdown of Ar-Rsk2 resulted in decreased levels of cyclin D3 and phosphorylated histone H3, and the production of pseudo-diapause cysts. This indicates that Ar-Rsk2 regulates mitotic arrest. PLK and ERK RNAi showed that Ar-Rsk2, but not Ar-Rsk1, could be activated by PLK-ERK in Artemia. This is the first study to report that RSK isoforms with and without an ERK-docking motif regulate mitosis and meiosis, respectively. This study provides insight into the relationship between the structures and functions of RSK isoforms.

The ribosomal S6 kinase (RSK) family comprises a group of ERK/MAPK effectors that regulate diverse cellular functions by phosphorylating nuclear and cytoplasmic targets. Hyperactive RSK signaling regulates cell division in several types of cancer, including breast, prostate, and lung (1–3). The RSK inhibitor SL-0101 arrests MCF-7 cells (a breast cancer cell line) in the G1 phase, and thereby inhibits their proliferation (1). RSK is overexpressed in more than 50% of primary malignant lung lesions, whereas it is undetectable in normal epithelium (3). This suggests that RSK promotes the proliferation of tumor cells.

In most metazoa, unfertilized oocytes are arrested at meiotic metaphase because of a cytostatic activity called cytostatic factor (CSF) (4, 5). This ensures that oocytes are released into the ovisac (uterus) in an optimal physiological state, which reduces the risk of insemination failing, such as occurs in polyspermy (6). The E3 ubiquitin ligase anaphase-promoting complex/cyclosome can degrade the components of CSF, after which meiosis resumes (4). The MAPK-RSK pathway is required to maintain CSF-dependent meiotic arrest. In this pathway, RSK phosphorylates its substrate Erp1 (also called Emi2) onSer335-Thr336, which leads to activation of Erp1. Activated Erp1 inhibits anaphase-promoting complex/cyclosome in vivo and in vitro (7, 8).

RSK isoforms contain an N-terminal kinase domain (NTKD) and a C-terminal kinase domain (CTKD), which are connected by a regulatory linker. All RSK isoforms, including the orthologues of RSK in Caenorhabditis elegans and Drosophila melanogaster, contain four essential phosphorylation sites (Ser221, Ser363, Ser380, and Thr573 in human Rsk1) that are responsive to mitogenic stimuli (9). ERK1/2 phosphorylates Thr573 (located in the CTKD) and Ser363 by docking to the ERK-docking motif. The CTKD then autophosphorylates Ser-380, which creates a docking site for PDK1, which, in turn, phosphorylates Ser221 in the NTKD. Phosphorylation of Ser221 stabilizes NTKD in an active conformation, resulting in full activation of RSK (9–11). This hierarchical phosphorylation cascade is essential to allow active RSK isoforms to perform their various functions.
The ERK-docking motif \((^{T39L}AQRRVRKLPSTTL^{T252})\) is located near the C terminus of RSK and proves an important motif for RSK activation. In quiescent HEK293 cells, endogenous ERK1/2 associates with immunoprecipitated wild-type Rsk1. However, a C terminally truncated Rsk1, lacking the last 11 amino acids that encode the ERK-docking motif, does not associate with ERK1/2 (12). Roux et al. (12) transfected HEK293 cells with Rsk1 point mutants and found that the Rsk1 L739A, R742A, and R743A mutants were unable to bind ERK1/2. These mutations also prevented Rsk1 activation, as shown by kinase assays using substrates for the N-terminal kinase activity of Rsk1 (12). Therefore, the ERK-docking motif is required for ERK1/2 binding to RSK and is shown to be essential for ERK1/2-mediated activation.

The effects elicited by RSK isoforms depend on the cell type and isoform. Cell proliferation is promoted by some RSK isoforms, whereas it is inhibited by others (13–18). Rsk1 and Rsk2 positively regulate the proliferation of tumor cells (1, 13). Consistent with this, the level of Rsk1 is often higher in tumors than in healthy tissues (14). By contrast, levels of Rsk3 and Rsk4 are usually lower in tumors than in healthy tissues (15). In addition, Rsk4 plays an inhibitory role in embryogenesis (15), and participates in p53-dependent cell growth arrest (16) and oncogene-induced cell senescence (17). Rsk2 is highly expressed in *Xenopus* oocytes and helps control critical stages of the meiotic cell cycle (18). For example, Rsk2 controls G2/M progression in meiosis I by phosphorylating and thereby inhibiting the kinase Myt1 (19). Rsk1 is thought to promote metaphase II arrest by phosphorylating and thereby activating the kinase Bub1, which is a mediator of anaphase-promoting complex inhibition (20).

Salt lakes on plateaus are the most hostile environments on earth, and hardly any animals can survive in these environments. One notable exception is *Artemia*, a small crustacean that can survive in extreme conditions such as high salinity, low levels of oxygen, and large change in temperature (21). To cope with such harsh habitats and widely different environmental conditions, *Artemia* has two independent reproductive pathways (22). Under unfavorable environmental conditions, mature *Artemia* produce and release encysted gastrula embryos that follow the diapause-directed developmental pathway and thereby enter diapause (an obligate dormancy) (23, 24). When environmental conditions are favorable, embryonic development proceeds uninterrupted, and mature *Artemia* release swimming nauplius larvae.

Diapause-directed development can be divided into four stages, namely, pre-diapause, diapause, post-diapause, and nauplius. In the pre-diapause stage, fertilized embryos develop into late gastrulae in the uterus (21). Diapause embryos are composed of 4000 cells arrested at G1/S phase. In these embryos, metabolic activity is greatly reduced and RNA/protein synthesis does not occur (25–28). Post-diapause embryos, in which the cell cycle is arrested at the G2/M phase (29), are obtained by storing diapause embryos at \(-20 °C\) for at least 3 months (30). In favorable conditions, post-diapause embryos hatch, the diapause is terminated, and cell division and development resume, resulting in the generation of nauplii (30–32). The mechanisms underlying this development remain unclear. A complex enzymatic system, including the RSK regulatory pathway, is suggested to be involved in diapause formation and termination (30, 33, 34).

In this study, we identified a novel RSK isoform in *Artemia*, which we termed Ar-Rsk2. We compared this function of this kinase with that of another RSK isoform that we previously identified called Ar-Rsk1. Ar-Rsk1, which lacks an ERK-docking motif, played an important role in regulating CSF and mitotic arrest. By contrast, Ar-Rsk2, which has an ERK-docking motif, functioned in the regulation of mitotic arrest. This is the first study to report that RSK isoforms with and without an ERK-docking motif regulate mitosis and meiosis, respectively. Thus, this study provides an insight into the relationship between the structures and functions of RSK isoforms.

### EXPERIMENTAL PROCEDURES

**Animals**—Specimens of amphigenic *Artemia franciscana* from salterns in San Francisco Bay were purchased from San Francisco Bay Brand, Hayward, CA, as dried (activated) encysted embryos. The daily light regime (h of light:h of dark) and the percentage of artificial seawater determine the reproductive mode of these animals (36). Diapause-destined *Artemia* were raised in 8% (w/v) artificial seawater at 25 °C in a 4-h light:20-h dark cycle, whereas *Artemia* that developed directly were raised in 4% (w/v) artificial seawater in a 16-h light:8-h dark cycle. Chlorella powder was supplied as brine shrimp food.

**Cloning of Ar-Rsk1 and Ar-Rsk2**—Ar-Rsk1 cDNA was isolated as described by Dai et al. (25). Ar-Rsk2 cDNA was obtained by performing two rounds of 3′ rapid amplification using the FirstChoice\textsuperscript{TM} RLM-RACE Kit and 5′ PCR with gene-specific primers (Table 1). The sequence of Ar-Rsk2 was imported into SeqMan Lasergene software. The sequences of the complete cDNA and the deduced peptide were determined using Blastn and Blastx, respectively, on the NCBI website. The nucleotide sequence of *Ar-Rsk2* cDNA was submitted to GenBank\textsuperscript{TM} under accession number KJ679441.

**Semi-quantitative RT-PCR**—Total RNA was extracted using TRIzol reagent according to the manufacturer’s instructions. First-strand cDNA was synthesized from 1 μg of total RNA using M-MLV Reverse Transcriptase in a 10-μl reaction. Ar-Rsk1, Ar-rsk2, and α-tubulin cDNAs (GenBank accession number AF427598) were amplified separately in 25-μl reactions using 1 μl of the cDNA as a template. The primers used are shown in Table 1.

**dsRNA Synthesis and Microinjection**—Reconstructed plasmids that contained two inverted T7 polymerase sites flanking the cloning region were obtained (named pET-T7), as described previously (25). The 3′ terminus differs between Ar-Rsk1 and Ar-Rsk2. A 249-bp fragment of Ar-Rsk1 and a 171-bp fragment of Ar-Rsk2, including the 3′-UTR and parts of the 3′ coding region, were PCR amplified using the Ar-Rsk1f/Ar-Rsk1r and Ar-Rsk2f/Ar-Rsk2r primer sets, respectively (Table 1). These amplified fragments were digested with XbaI and EcoRI and subcloned into pET-T7. For the negative control, a 359-bp fragment of *GFP* was amplified from the pcDNA3.1/CT-GFP-TOPO\textsuperscript{®} plasmid (using primers GFPf and GPFr; Table 1) and subcloned into pET-T7 between the XbaI and NcoI restriction sites. These recombinant plasmids were used to express dsRNA of GFP, Ar-Rsk1, and Ar-Rsk2.
After transformation into *Escherichia coli* strain DH5α, the sequences of the inserted fragments in the plasmids were confirmed by DNA sequencing. The recombinant plasmids were firmed by DNA sequencing. The recombinant plasmids were transformed into the E. coli strain HT115, and dsRNAs were purified as described by Yodmuang et al. (37). dsRNA was microinjected into *Artemia* using the Ultra-MicroPump II equipped with a Microport MicroSyringe pump controller. One μg of dsRNA was injected per adult, prior to the formation of oocytes (34).

**TABLE 1**
**Primer sequences used for PCR**

The sequences that are recognized by restriction endonucleases are underlined. TubulinF and TubulinR were designed based on the cDNA sequence stored under GenBank™ accession number AF247598 (R, reverse; F, forward).

| Primer      | Length | Positions of nucleotides | Direction | Sequence (5′ – 3′) |
|-------------|--------|--------------------------|-----------|--------------------|
| 3′ F1       | 20     | 1772–1791                | F         | TGGCACTTTGGTTTCCGAAA |
| 3′ F2       | 20     | 1820–1839                | F         | AGCGCTGTTGCTAACTGCCC |
| 5′ F        | 24     | 1–24                     | F         | ACGTTCTTACGAAATTGAGCTC |
| 5′ R        | 20     | 1875–1906                | R         | TTTCCTCGAAATGTGCTAGC |
| Ar-Rsk1QF   | 21     | 2212–2234                | F         | TAAATTGTAGACCATGAGGAC |
| Ar-Rsk1QR   | 23     | 2428–2450                | F         | CGAAGAAATTTCACCGGTTG |
| Ar-Rsk2QF   | 23     | 2208–2230                | R         | TGCTGTGCCTTTACCACTTCTAGT |
| Ar-Rsk2QR   | 23     | 2558–2580                | F         | CGATCTGCTCACATTTACCTCGT |
| TubulinF    | 20     | 446–465                  | F         | GCTAGTGTCTACTACAGGTTTTC |
| TubulinR    | 21     | 777–795                  | R         | ATCCAACCAAAGGTCTGCGG |
| Ar-Rsk1F    | 27     | 2217–2235                | F         | GCTCTTAATATTATGACGCA |
| Ar-Rsk1R    | 27     | 2246–2265                | R         | GAAATATGCTGCCTACATCTAGT |
| Ar-Rsk2F    | 28     | 2210–2229                | F         | GCTTTCAATCTGACCCTAATTCCCA |
| Ar-Rsk2R    | 30     | 2358–2380                | R         | GAATTTCCAGACCTGACAACCTACCTCGA |
| GFPiF       | 30     | 122–144                  | F         | GCTTCTAATGCTTTACCATTTTATTGTC |
| GFPiR       | 28     | 461–480                  | R         | CAACCCCTAACCTTTGCTTCCTC |
| Ar-Rsk1EF   | 25     | 1785–1812                | F         | CATTTACACAAAGAAAAACGCT |
| Ar-Rsk1ER   | 26     | 2215–2240                | R         | AGATTCGCTTCTCTGGAATCAATATAT |
| Ar-Rsk2EF   | 20     | 1886–1905                | F         | TGATGACATTGGCACCTTGG |
| Ar-Rsk2ER   | 25     | 2274–2298                | R         | GTTGGATCAGTGACTGCTCCCTT |

**Co-immunoprecipitation**—Samples were stirred in FA lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors, and 40 units/ml of RNasin) on ice for 15 min. Lysates were incubated with Protein A Dynabeads for 1–2 h, and then clarified by centrifugation. Supernatants were incubated overnight at 4 °C with anti-Ar-Rsk1 or anti-Ar-Rsk2 antibodies or with chitin-binding protein antibody as a control. Beads were washed with extraction buffer twice and then mixed with 2× loading buffer (0.125 x Tris, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.02% bromphenol blue). Samples were boiled, separated by SDS-PAGE, and Western blotted.

**Phosphorylation Assay of Ar-Rsk1 and Ar-Rsk2**—Immuno-precipitation was performed using pan-antibodies that recognized both non-phosphorylated and phosphorylated forms of Ar-Rsk1 and Ar-Rsk2. Western blotting of these immunoprecipitates was performed using these pan-antibodies or with antibodies that specifically recognized phosphorylated forms of Ar-Rsk1 and Ar-Rsk2 (Ser219 or Ser380). When a similar amount of total Ar-Rsk1 or Ar-Rsk2 was immunoprecipitated, the level of phosphorylated protein reflected the activity of the given isoform.

**BrdU Incorporation Assay—Artemia** were incubated in seawater containing 1 mM BrdU for 24 h, fixed with 4% (w/v) paraformaldehyde, and paraffin-embedded. Ten-μm thick tissue sections were incubated with a mouse monoclonal anti-BrdU antibody at 4 °C overnight, and then with alkaline phosphatase-conjugated anti-mouse IgG antibody. Staining was performed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution in the dark. The staining reaction was stopped by the addition of 10 mM TE buffer (10 mM Tris-Cl, pH 8.0, and 1 mM EDTA).

**RESULTS**

**Characterization of Meiotic and Mitotic Arrest during Artemia Development**—The oviparous and ovoviviparous life cycles of *Artemia* were controlled by modulating the different percentage of seawater and the daily light regime of the animals.
Involvement of Ar-RSKs in Meiotic and Mitotic Arrest

Identification of Two RSK Isoforms in Artemia—We studied the functions of RSK isoforms in the regulation of meiotic and mitotic arrest in Artemia. We previously reported that an Ar-RSK isoform, termed Ar-Rsk1, plays an important role in diapause termination in Artemia (29). This isoform lacks a typical ERK-docking motif. In the current study, we identified another Ar-RSK isoform in Artemia, termed Ar-Rsk2, which has an ERK-docking motif. Sequence analysis revealed that the cDNA of Ar-Rsk2 contains an open reading frame that encodes a protein of 734 aa, with a predicted mass of 83.7 kDa (Fig. 2A). The deduced aa sequence of this protein is similar to those of other RSK family members. Ar-Rsk2 has a 98% sequence identity with Ar-Rsk1 (Fig. 2B), 59% with human and mouse Rsk1/2, 60% with human and mouse Rsk3, and 58% with human Rsk4. Phylogenetic analysis suggested that Ar-Rsk2 can be grouped with the RSK expressed in D. melanogaster (56% sequence identity) (Fig. 2D).

To address the genomic aspects of Ar-Rsk1 and Ar-Rsk2, we enzyme digested the genomic DNA with BamHI, EcoRI, KpnI, and XbaI and then incubated the enzyme-digested product with a probe covering the common fragments of Ar-Rsk1 and Ar-Rsk2. The results of Southern blot showed that only one band in each enzyme lane had been hybridized by the probe (Fig. 2E). This indicated that Ar-Rsk1 and Ar-Rsk2 are spliced from one unique single-copy gene.

The deduced aa sequence showed that Ar-Rsk2 has two distinct kinase domains, NTKD and CT KD, which are joined by a linker region (Fig. 2C). This is identical to the structure of Ar-Rsk1. Ar-Rsk2 contains five phosphorylation sites (Ser218, Thr359, Ser363, Ser380, and Thr571), which were identified according to RSK consensus phosphorylation sites (9). However, aa 720–724 of Ar-Rsk2 (Leu-Ala-Lys-Arg-Arg) constitute an ERK-docking site (14), which Ar-Rsk1 lacks (Fig. 2, B and C).

Expression Pattern of Ar-Rsk1 and Ar-Rsk2 during Diapause Formation and Termination—Semi-quantitative RT-PCR showed that Ar-Rsk1 was stably expressed throughout Artemia development (Fig. 3, A and B). The level of Ar-Rsk2 mRNA was high in early embryos, nauplii, and metanauplii (Fig. 3, A and B), in which cells are dividing. However, expression of Ar-Rsk2 was suppressed in diapause and post-diapause embryos (Fig. 3B), in which cells are in mitotic arrest.

Using polyclonal antibodies specific to Ar-Rsk1 or Ar-Rsk2, the bands of 81 and 83 kDa were identified in Artemia, respectively. The results of the Western blot indicated that Ar-Rsk1 was expressed in all tested tissues, with the exception of those of the cephalothorax and embryos during embryonic development. The expression of Ar-Rsk2 was specific to the oocyte-containing or embryo-containing abdomen of Artemia (Fig. 3C). The level of Ar-Rsk1 expression remained constant throughout development (Fig. 3, D and E), however, Ar-Rsk2 expression was reduced in pre-diapause embryos, and was suppressed in diapause and post-diapause embryos. Thereafter, Ar-Rsk2 expression gradually increased and was highest in nauplii, in which cells are dividing (Fig. 3, D and E).

The consensus phosphorylation sites of Ar-Rsk1 and Ar-Rsk2 were mapped (Fig. 2B). Immunoprecipitations were performed using polyclonal antibodies that specifically recognized Ar-Rsk1 or Ar-Rsk2. Western blotting of these immuno-
Involvement of Ar-RSKs in Meiotic and Mitotic Arrest

A

B

C

D

E
precipitates was performed using antibodies that specifically recognized Ar-Rsk1 and Ar-Rsk2 phosphorylated at Ser218 (Ser\textsuperscript{218}) in human Rsk1 and Ar-Rsk2, respectively. The results indicated that Ar-Rsk1 and Ar-Rsk2, F, the specific phosphorylation levels of Ar-Rsk1 each in cephalothorax, abdomen, or embryos. G and H, levels of phosphorylated Ar-Rsk1 during Artemia development. I, the specific phosphorylation level of Ar-Rsk2 each in cephalothorax, abdomen, or embryos. J and K, levels of phosphorylated Ar-Rsk2 during Artemia development. Lane 1, early oogenesis; lane 2, late oogenesis; lane 3, early embryos; lane 4, pre-diapause embryos; lane 5, diapause embryos; lane 6, post-diapause embryos; lane 7, 8-h incubated embryos; lane 8, nauplii; lane 9, metanauplii. P, positive control (protein sample from late oogenesis). ce, cephalothorax; ab, abdomen; em, embryos.

Ar-Rsk2 was higher in early embryos than in oocytes (Fig. 3J). The level of phosphorylated Ar-Rsk2 was suppressed in diapause and post-diapause embryos and resumed after diapause termination (Fig. 3A). These results indicate that Ar-Rsk2 is involved in mitotic arrest during early embryonic development.

Ar-Rsk1 Controls Meiotic Arrest during Oogenesis in Artemia—To further study the functions of Ar-RSKs in meiotic arrest, Ar-Rsk1 or Ar-Rsk2 was knocked down in immature Artemia using RNAi. Protein levels of Ar-Rsk1 and Ar-Rsk2 were reduced by more than 90% following injection of Ar-Rsk1 dsRNA or Ar-Rsk2 dsRNA, respectively (Fig. 4A). The signals of Ar-Rsk1 in the Ar-Rsk2 RNAi and Ar-Rsk2 in the Ar-Rsk1 RNAi were reduced by more than 90% following injection of Ar-Rsk1 dsRNA (Ar-Rsk1i), or Ar-Rsk2 dsRNA (Ar-Rsk2i). These results indicate that Ar-Rsk2 is involved in meiotic arrest during oogenesis in Artemia.
change during late oogenesis following knockdown of Ar-Rsk1, whereas the level of phospho-cdc2 (Tyr15) was clearly decreased (Fig. 4C). Phosphorylation and activation of cdc2 are necessary to form the active cdc2-cyclin B1 complex. These results indicate that phosphorylated Ar-Rsk1 is involved in the formation and activation of this complex, which arrests oocytes in meiosis. By contrast, the levels of total cdc2, phospho-cdc2 (Tyr15), and cyclin B1 did not change during late oogenesis following knockdown of Ar-Rsk2 (Fig. 4D), indicating that Ar-Rsk2 is not involved in the initiation or maintenance of meiotic arrest.

Following knockdown of Ar-Rsk1, typically malformed nauplii were produced with various uromere deformities and shortened swimming setae (Fig. 4E, b). Nauplii produced by GFP dsRNA-injected Artemia did not exhibit these malformations (Fig. 4E, a). These malformed nauplii could not swim normally and most died within 1 week. Individuals that had been injected with Ar-Rsk2 dsRNA released pseudo-diapause cysts (Fig. 4E, c). These did not develop into nauplii. Taken together, these data indicate that Ar-Rsk1 is important for oogenesis and the development of embryos after fertilization, whereas Ar-Rsk2 is involved in the development of embryos.

Ar-Rsk2 Controls Mitotic Arrest during Embryo Development in Artemia—To investigate the functions of Ar-Rsk1 and Ar-Rsk2 in the regulation of mitotic arrest during the development of Artemia, Ar-Rsk1 dsRNA or Ar-Rsk2 dsRNA was injected during the early stages of embryo development, after meiosis was completed. Ar-Rsk2 was weakly expressed in diapause and post-diapause embryos; therefore, we performed RNAi in Artemia that were following the ovoviviparous (directly developing) pathway.

Western blot analysis showed that the protein levels of Ar-Rsk1 were reduced by more than 80% and those of Ar-Rsk2 were reduced by more than 90% (Fig. 5A) following injection of Ar-Rsk1 or Ar-Rsk2 dsRNA, respectively. The expression of neither Ar-Rsk1 in the Ar-Rsk1 RNAi nor Ar-Rsk2 in the Ar-Rsk2 RNAi was affected following knockdown (Fig. 5B). Up to 90% of individuals injected with Ar-Rsk2 dsRNA released pseudo-diapause cysts, whereas individuals injected with GFP or Ar-Rsk1 dsRNA released nauplii (Fig. 5C). Furthermore, the BrdU assay showed that following Ar-Rsk2 knockdown, cells did not divide and embryos did not develop (Fig. 5D). By contrast, following injection of GFP dsRNA or Ar-Rsk1 dsRNA, cell division occurred normally and embryos developed to the blastula stage (Fig. 5D).

Western blot analysis showed that levels of cyclin D3 and phospho-histone H3 (Ser10) were decreased following knockdown of Ar-Rsk2 (Fig. 5F), whereas they were not changed following knockdown of Ar-Rsk1 (Fig. 5E). These results indicate that Ar-Rsk2 is involved in regulation of mitosis and plays important roles in diapause formation in Artemia. In summary, Ar-Rsk2 functions in diapause formation by regulating mitotic arrest, whereas Ar-Rsk1 functions in oocyte development by regulating meiotic arrest.

DISCUSSION

Artemia is a useful model in which to study cell cycle arrest because cells arrest in meiosis and mitosis during oogenesis and...
embryonic development, respectively. In the present study, we found that Ar-Rsk1 and Ar-Rsk2 have different expression patterns and levels of phosphorylation during development, and play markedly different roles in meiotic and mitotic arrest.

RSK isoforms are emerging as multifunctional effectors that have distinct functions. Ectopic expression of Rsk2 increases the proliferation rate and anchorage-independent transformation of mouse embryonic fibroblasts (38). Rsk1 and Rsk2 may also promote G1 phase progression by controlling the activity of p27kip1, a cyclin-dependent kinase 2 inhibitor (39). However, Rsk3 was recently shown to act as a tumor suppressor in ovarian cancer (40), and several lines of evidence suggest that Rsk4 negatively regulates cell proliferation (41). The current study shows that RSK isoforms also have distinct functions in Artemia; Ar-Rsk1 regulates meiosis, whereas Ar-Rsk2 regulates mitosis.

RSK isoforms interact with ERK through their C-terminal docking domains, which have an ERK-docking motif consensus sequence (42). This motif corresponds to a hydrophobic residue closely followed by two lysine or arginine residues, which are positively charged (Leu-Xaa2-Lys/Arg-Lys/Arg-Xaa5-Leu). Although this motif is not conserved in RSK orthologues from various species, it is required for RSK activation (43). Like the case of Ar-Rsk1 in Artemia, five human RSK δ-1 proteins (NP_036556, NP_001129610, NP_001274148, NP_001274149, and NP_001274150) and a Xenopus RSK δ-1 protein (XP_002934734) all belong to the RSK family, but lack a typical ERK-docking domain. Based on our results, these RSKs could not be activated by PLK and ERK pathways, however, the mechanism of activation of the RSKs without the ERK-docking motif remains large unclear. In the present study, the function of Ar-Rsk1 was shown to control meiotic arrest during oogenesis. Thus, the biological function of these RSKs from other species without the ERK-docking motif is considered involved in the regulation of meiosis during oogenesis.

RSKs are involved in the regulation of cell proliferation in various malignancies and genetic diseases by directly or indirectly modulating the cell cycle machinery (44–46). We report that Ar-Rsk2 promotes cell proliferation during early embryonic development in Artemia by regulating the cell cycle. These findings are consistent with reports that all RSK isoforms (except Rsk4) are ubiquitously expressed at the mRNA level in regions that contain rapidly proliferating cells in mice and humans (14).

Artemia that developed via the oviviparous pathway were extremely sensitive to knockdown of Ar-Rsk2 on mitosis at early stages of development. These effects were more pronounced than those reported by Smith et al. (1) in the human breast cancer cell line MCF-7; proliferation of these cells is suppressed following treatment with an inhibitor of RSK function or knockdown of RSK expression. We propose the novel hypothesis that RSK isoforms specifically regulate mitosis in early embryonic cells and in other distinct types of cells, such as tumor-initiating cells. This may explain why triple-negative breast cancers, which contain a higher proportion of tumor-initiating cells than other breast cancer subtypes, are particularly sensitive to RSK inhibition (47). However, this hypothesis needs to be investigated further.

Extensive study of meiotic arrest during oocyte maturation revealed that the MAPK-RSK pathway is essential for CSF activity (8, 48). Little is known about CSF-dependent meiotic arrest in invertebrate species (49, 50). In Artemia, oocytes are arrested in metaphase I until fertilization occurs (22). Ar-Rsk1 was phosphorylated and active when CSF activity was high, whereas it was dephosphorylated when meiosis resumed (Fig. 3). Knockdown of Ar-Rsk1 resulted in dephosphorylation of cdc2 in oocytes and termination of meiotic arrest (Fig. 5). Most of these oocytes completed meiosis, and embryo development proceeded normally to produce nauplii. However, some of these nauplii exhibited malformations, such as shortened swimming setae and a jagged uroome. These malformations reflect the biological importance of meiotic arrest in oocytes; this ensures that oocytes are released into the ovisac (uterus) in the optimal physiological state, which prevents insemination failure or occurring abnormally. Our findings show that the Ar-Rsk1 pathway participates in maintaining the CSF-dependent meiotic arrest in Artemia, which is required for oocyte

**FIGURE 6.** The identification of the signals triggering Ar-Rsk1 and Ar-Rsk2 activation. A, the expression of Ar-Rsk1 and Ar-Rsk2 following injection of GFP dsRNA (GFPi), ERK dsRNA (ERKi), or PLK dsRNA (PLKi). B, the phosphorylation level of Ar-Rsk1 and Ar-Rsk2 following injection of GFP dsRNA (GFPi), ERK dsRNA (ERKi), or PLK dsRNA (PLKi). α, the phosphorylation level of Ar-Rsk1 and Ar-Rsk2 following ERKi. β, the phosphorylation level of Ar-Rsk1 and Ar-Rsk2 following PLKi.
maturation. These results highlight the complexity of meiotic regulation, which has evolved to ensure that reproduction is precisely controlled.

RSK isoforms control cell proliferation by regulating mediators of the cell cycle (51, 52). RSK with ERK-docking motif phosphorylates src on Tyr and Thr residues (52). This inhibits and inactivates Myt1, a membrane-associated kinase that phosphorylates cdc2 on Tyr and Thr residues (52). This inhibits cdc2, leading to cell cycle progression. This is the first study to report that RSK isoforms with and without an ERK-docking motif regulate mitosis and meiosis, respectively. This study provides insight into the relationship between the structures and functions of RSK isoforms.

REFERENCES

1. Smith, J. A., Poteet-Smith, C. E., Xu, Y., Errington, T. M., Hecht, S. M., and Lannigan, D. A. (2005) Identification of the first specific inhibitor of p90 ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. Cancer Res. 65, 1027–1034
2. Imada, K., Shiota, M., Kohashi, K., Kuroiwa, K., Song, Y., Sugimoto, M., Naito, S., and Oda, Y. (2013) Mutual regulation between Raf/MEK/ERK signaling and Y-box-binding protein-1 promotes prostate cancer progression. Clin. Cancer Res. 19, 4638–4650
3. Lara, R., Mauri, F. A., Taylor, H., Derua, R., Shia, A., Gray, C., Nicolas, A., Shiner, R. J., Schofield, E., Bates, P. A., Waelkens, E., Dallman, M., Lamb, J., Zicha, D., Downward, J., Seckl, M. J., and Pardo, O. E. (2011) An siRNA screen identifies RSK1 as a key modulator of lung cancer metastasis. Oncogene 30, 3513–3521
4. Schmidt, A., Rauh, N. R., Nigg, E. A., and Mayer, T. U. (2006) Cytostatic factor: an activity that puts the cell cycle on hold. J. Cell Sci. 119, 1213–1218
5. Liu, J., Grimson, B., and Maller, J. L. (2007) New insight into metaphase arrest by cytostatic factor: from establishment to release. Oncogene 26, 1286–1289
6. Moriwaki, K., Nakagawa, T., Nakaya, F., Hirohashi, N., and Chiba, K. (2013) Arrest at metaphase of meiosis I in starfish oocytes in the ovary is maintained by high CO₂ and low O₂ concentrations in extracellular fluid. Zool. Sci. 30, 975–984
7. Tunquist, B. J., and Maller, J. L. (2003) Under arrest: cytostatic checkpoint kinase Bub1 and cyclin E/Cdk2 both contribute to the establishment of meiotic metaphase arrest by cytostatic factor. Curr. Biol. 12, 1027–1033
8. Liu, Y. L., Zhao, Y., Dai, Z. M., Chen, H. M., and Yang, W. J. (2009) Formation of diapause cyst shell in brine shrimp, Artemia parthenogenetica, and its resistance role in environmental stresses. J. Biol. Chem. 284, 16931–16938
9. Abatzopoulos, T. H. J., Beardmore, J. A., Clegg, J. S., Sorgeloos, P. (eds) (2002) Artemia: Basic and Applied Biology, Kluwer Academic Publishers, Dordrecht, The Netherlands
10. Chen, S., Chen, D. F., Yang, F., Nagasawa, H., and Yang, W. J. (2011) Characterization and processing of superoxide dismutase-fused vitellogenin in the diapause embryo formation: a special developmental pathway in the brine shrimp, Artemia parthenogenetica. Biol. Reprod. 85, 31–41
11. Ma, W. M., Li, H. W., Dai, Z. M., Yang, J. S., Yang, F., and Yang, W. J. (2013) Chitin-binding proteins of Artemia diapause cysts participate in formation of the embryonic cuticle layer of cyst shells. Biochem. J. 449, 285–294
12. Dai, Z. M., Li, R., Dai, L., Yang, J. S., Chen, S., Zeng, Q. G., Yang, F., and Yang, W. J. (2011) Determination in oocytes of the reproductive modes for the brine shrimp Artemia parthenogenetica. Biosci. Rep. 31, 17–30
13. Zhu, X. J., Dai, J. Q., Tan, X., Zhao, Y., and Yang, W. J. (2009) Activation of an AMP-activated protein kinase is involved in post-diapause development of Artemia franciscana encysted embryos. BMC Dev. Biol. 9, 21
14. Clegg, J. S. (1978) Interrelationships between water and cellular metabolism in Artemia cysts. VIII Sorption isotherms and derived thermodynamic quantities. J. Cell. Physiol. 94, 123–137
15. Clegg, J. (1997) Embryos of Artemia franciscana survive four years of continuous anoxia: the case for complete metabolic rate depression. J. Exp. Biol. 200, 467–475
16. Dai, J. Q., Zhu, X. J., Liu, F. Q., Xiang, J. H., Nagasawa, H., and Yang, W. J. (2008) Involvement of p90 ribosomal S6 kinase in termination of cell cycle arrest during development of Artemia-encysted embryos. J. Biol. Chem. 283, 1705–1712
17. Clegg, J. S. (2007) Protein stability in Artemia embryos during prolonged anoxia. Biol. Bull. 212, 74–81
18. Zhou, R., Yang, F., Chen, D. F., Sun, Y. X., Yang, J. S., and Yang, W. J. (2013) Acetylation of chromatin-associated histone H4 lysine 56 inhibits the development of encysted Artemia embryos. PLoS ONE 8, e68374
19. Feng, C. Z., Zhu, X. J., Dai, Z. M., Liu, F. Q., Xiang, J. H., and Yang, W. J. (2007) Identification of a novel DNA methyltransferase 2 from the brine shrimp, Artemia franciscana. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 147, 191–198
20. Yang, F., Jia, S. N., Yu, Y. Q., Ye, X., Liu, J., Qian, Y. Q., and Yang, W. J. (2012) Deubiquitinating enzyme BAP1 is involved in the formation and maintenance of the diapause embryos of Artemia. Cell Stress Chaperones 17, 577–587
34. Li, R., Chen, D. F., Zhou, R., Jia, S. N., Yang, J. S., Clegg, J. S., and Yang, W. J. (2012) Involvement of polo-like kinase 1 (Plk1) in mitotic arrest by inhibition of mitogen-activated protein kinase-extracellular signal-regulated kinase-ribosomal S6 kinase 1 (MEK-ERK-RRSK1) cascade. *J. Biol. Chem.* 287, 15923–15934

35. Rath, A., Hromas, R., and De Benedetti, A. (2014) Fidelity of end joining in mammalian epithomes and the impact of Notase on joint processing. *BMC Mol. Biol.* 15, 6

36. Nambu, Z., Tanaka, S., and Nambu, F. (2004) Influence of photoperiod and temperature on reproductive mode in the brine shrimp, *Artemia franciscana*. *J. Exp. Zool. A Comp. Exp. Biol.* 301, 542–546

37. Yodmuang, S., Tirasophon, W., Roshorm, Y., Chinnirunvong, W., and Panyim, S. (2006) YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. *Biochem. Biophys. Res. Commun.* 341, 351–356

38. Cho, Y. Y., Yao, K., Kim, H. G., Kang, B. S., Zheng, D., Bode, A. M., and Dong, Z. (2007) Ribosomal S6 kinase 2 is a key regulator in tumor promoter-induced cell transformation. *Cancer Res.* 67, 8104–8112

39. Fujita, N., Sato, S., and Tsuruo, T. (2003) Phosphorylation of p27kip1 at threonine 198 by p90 ribosomal protein S6 kinase promotes its binding to 14–3-3 and cytoplasmic localization. *J. Biol. Chem.* 278, 49254–49260

40. Bignone, P. A., Lee, K. Y., Liu, Y., Emilion, G., Finch, J., Soosay, A. E., Charnock, F. M., Beck, S., Dunham, I., Mungall, A. J., and Ganesan, T. S. (2008) RPS6KA2, a putative tumour suppressor gene at 6q27 in sporadic epithelial ovarian cancer. *Oncogene* 26, 683–700

41. Thakur, A., Sun, Y., Bollig, A., Wu, J., Biliran, H., Banerjee, S., Sarkar, F. H., and Liao, D. J. (2008) Anti-invasive and antimetastatic activities of ribosomal protein S6 kinase 4 in breast cancer cells. *Clin. Cancer Res.* 14, 4427–4436

42. MacKenzie, S. J., Baillie, G. S., McPhee, I., Bolger, G. B., and Houslay, M. D. (2000) ERK2 mitogen-activated protein kinase kinase binding, phosphorylation, and regulation of the PDE4D cAMP-specific phosphodiesterases: the involvement of COOH-terminal docking sites and NH2-terminal UCR regions. *J. Biol. Chem.* 275, 16609–16617

43. Smith, J. A., Poteet-Smith, C. E., Malarkey, K., and Sturgill, T. W. (1999) Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK in vivo. *J. Biol. Chem.* 274, 2893–2898

44. Neise, D., Sohn, D., Stefanski, A., Goto, H., Inagaki, M., Wesselborg, S., Budach, W., Stühler, K., and Jänicke, R. U. (2013) The p90 ribosomal S6 kinase (RSK) inhibitor BI-D1870 prevents gamma irradiation-induced apoptosis and mediates senescence via RSK- and p53-independent accumulation of p21waf1/cip1. *Cell Death Dis.* 4, e859–e859

45. Arul, N., and Cho, Y. Y. (2013) A rising cancer prevention target of RSK2 in human skin cancer. *Front. Oncol.* 3, 201

46. Sulzmaier, F. J., and Ramos, J. W. (2013) RSK isoforms in cancer cell invasion and metastasis. *Cancer Res.* 73, 6099–6105

47. Stratford, A. L., Reipas, K., Hu, K., Fotovati, A., Brough, R., Frankum, J., Takhar, M., Watson, P., Ashworth, A., Lord, C. J., Lasham, A., Print, C. G., and Dunn, S. E. (2012) Targeting p90 ribosomal S6 kinase eliminates tumor-initiating cells by inactivating Y-box binding protein-1 in triple-negative breast cancers. *Stem Cells* 30, 1338–1348

48. Yamamoto, D. S., Tachibana, K., Sumitani, M., Lee, J. M., and Hat-skeyama, M. (2008) Involvement of Mos-MEK-MAPK pathway in cyto-static factor (CSF) arrest in eggs of the parthenogenetic insect, *Atha1ia rosae*. *Mech. Dev.* 125, 996–1008

49. Ivanovska, I., Lee, E., Kwan, K. M., Fenger, D. D., and Orr-Weaver, T. L. (2004) The Drosophila MOS ortholog is not essential for meiosis. *Curr. Biol.* 14, 75–80

50. Kondoh, E., Tachibana, K., and Deguchi, R. (2006) Intracellular Ca2+ increase induces post-fertilization events via MAP kinase dephosphorylation in eggs of the hydrozoan jellyfish *Cladonema pacificum*. *Dev. Biol.* 293, 228–241

51. Bruning, J. C., Gillette, J. A., Zhao, Y., Bjorbaeck, C., Kotzka, J., Knebel, B., Avci, H., Hanstein, B., Lingohr, P., Moller, D. E., Krone, W., Kahn, C. R., and Muller-Wieland, D. (2000) Ribosomal subunit kinase-2 is required for growth factor-stimulated transcription of the c-Fos gene. *Proc. Natl. Acad. Sci. U.S.A.* 97, 2462–2467

52. Palmer, A., Gavin, A. C., and Nebreda, A. R. (1998) A link between MAP kinase and p34cdc2/cyclin B during oocyte maturation: p90rib phosphorylates and inactivates the p34cdc2 inhibitory kinase Myt1. *EMBO J.* 17, 5037–5047