Involvement of p90 Ribosomal S6 Kinase in Termination of Cell Cycle Arrest during Development of Artemia-encysted Embryos

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Received for publication, September 19, 2007, and in revised form, November 1, 2007 Published, JBC Papers in Press, November 12, 2007 DOI 10.1074/jbc.M707853200

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Artemia has evolved a unique developmental pattern of encysted embryos to cope with various environmental threats. Cell divisions totally cease during the preemergence developmental stage from gastrula to prenauplius. The molecular mechanism of this, however, remains unknown. Our study focuses on the involvement of p90 ribosomal S6 kinase (RSK), a family of serine/threonine kinase that mediates signal transduction downstream of mitogen-activated protein kinase cascades, in the termination of cell cycle arrest during the post-embryonic development of Artemia-encysted gastrula. With immunochemistry, morphology, and cell cycle analysis, the identified Artemia RSK was established to be specifically activated during the post-embryonic and early larval developmental stages when arrested cells of encysted embryos resumed mitoses. In vivo knockdown of RSK activity by RNA interference, kinase inhibition, and antibody neutralization consistently induced defective larvae with distinct gaps between the exoskeleton and internal tissues. In these abnormal individuals, mitoses were detected to be largely inhibited in the affected regions. These results display the requirement of RSK activity during Artemia development and suggest its role in termination of cell cycle (G1/M phase) arrest and promotion of mitogenesis. Our findings may, thus, provide insights into the regulation of cell division during Artemia post-embryonic development and reveal further aspects of RSK functions.

p90 ribosomal S6 kinase (p90RSK, also known as RSK) is a family of serine/threonine kinase that mediates signal transduction downstream of mitogen-activated protein kinase cascades (1). RSKs2 consist of two distinct and functional kinase domains, the N-terminal kinase domain and the C-terminal kinase domain connected by a regulatory linker (2). The typical RSK possesses six phosphorylation sites, and its in vivo activation is achieved upon their consecutive phosphorylations. Mutation analysis reveals that four of the six sites are essential for the kinase activation (Ser-221 in N-terminal kinase domain, Ser-363 and Ser-380 in the linker, and Thr-573 in C-terminal kinase domain, according to the human RSK1 sequence) (3).

RSKs are present in most vertebrate species (1, 4), and RSK-related molecules have also been identified in several invertebrates (5, 6). RSK family members have been reported to be multifunctional in the regulation of diverse cellular processes including transcriptional regulation, cell cycle control, cell survival, and many others (4). Within mammalian cells, RSKs are thought to promote G1 progression by stimulating growth-related protein synthesis and inhibiting negative regulation (7). It is, thus, shown that the RSK inhibitor SL0101 can effectively inhibit proliferations of the human breast cancer cell and the hamster ovary cell (8, 9).

Artemia possesses powerful adaptations to extreme environments that often include aspects of hypersalinity, anoxia, and large changes in ionic composition and temperature. As a strategy to cope with such environmental stresses, Artemia has evolved a special reproductive mode in that in addition to giving birth to nauplii by the ovoviviparous pathway, this genus also releases encysted embryos by the oviparous pathway (10). Released encysted embryos are developmentally arrested at the gastrula stage with a low metabolic rate and complete turnoff of replication, transcription, and translation; however, without loss of embryonic viability (11, 12).

Another interesting phenomenon in the development of Artemia-encysted embryos is that no cell division or DNA synthesis occurs before emergence (13, 14). In the emergence process, the prenauplius starts to push out of the cyst shell, and finally, it develops into a true swimming nauplius. During preemergence development, the number of nuclei remains at about 4000 per embryo (15) despite the great number of internal events including deposited energy mobilization, RNA and protein synthesis restoration, cellular differentiation, and associated morphological changes required for the development from gastrula to prenauplius (16, 17). The mechanism of this unusual developmental pattern is still unclear. It is considered that the absence of cell division during the early embryonic development of Artemia is an adaptation to environmental threats, which confer on embryos their amazing stability and viability (18). In the present study Artemia RSK was established to be specifically activated at the very beginning of emergence and was
coupled with mitogenesis during the post-embryonic and early larval developmental stages. In vivo knockdown of RSK activity consistently induced abnormal individuals with distinct gaps between the exoskeleton and the internal tissues in the developing thoracic and abdominal regions. By 5-bromo-2'-deoxyuridine (BrdUrd) labeling and mitotic index analysis, mitoses were detected to be largely inhibited in those affected segments. Our findings, thus, indicate that RSK is involved in the termination of cell cycle (G2/M phase) arrest and the promotion of mitogenesis during the post-embryonic development of Artemia-encysted embryos.

EXPERIMENTAL PROCEDURES

Animals—Postdiapause embryos of Artemia parthenogenetica of Gahai were incubated in 2.5% artificial seawater (Blue Starfish, Hangzhou, Zhejiang, China) at 28 °C with continuous light after hydration at 4 °C for 5 h; swimming nauplii were cultured in 7% artificial seawater at 25 °C with a 16-h light cycle per day. After ovulation, 30% of the adult females were maintained in the same condition for producing nondiapause embryos. The other 70% were transferred to another culture condition with a 5-h light cycle per day for producing encysted embryos. Half of the gathered encysted embryos was stored at 15 °C as a sample set of diapause embryos, and the other half was dehydrated in saturated sodium chloride solution for 24 h and then stored at −20 °C to prepare a sample set of postdiapause embryos.

Molecular Cloning of RSK Encoding cDNA—The first-strand cDNAs of each sample were synthesized from 2 µg of total RNA using the SuperScript™ First-Strand cDNA Synthesis kit (Invitrogen). 2 µl of reverse transcription products were used as the PCR templates, and both the RSK cDNA fragment and the constitutively expressed α-tubulin cDNA fragment (GenBank™ accession number AF427598) were simultaneously amplified in a 25-µl reaction containing 10 pmol of F2 and R3 (Table 1) and 2 pmol of tubulinF and tubulinR (Table 1). Relative abundances were expressed as RSK transcript levels to those of α-tubulin. In each case peak values were set to 100, and the rest of the values were normalized. All data were given as the means ± S.E. of independent experiments from three separate RNA pools. All statistical analyses were performed using the one-way ANOVA, and the differences were considered significant for p < 0.01.

Western Blotting—Proteins were extracted from each sample using the TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions and were quantified using the Bradford method (20). 50 µg of proteins of each sample were separated on 10% SDS-PAGE and transferred to polyvinylidine difluoride membranes (Millipore). The membranes were incubated with anti-phospho-RSK antibody (phospho-p90RSK (Ser-380) antibody, Cell Signaling Technology, 1:1000) or anti-RSK antibody (rabbit anti-pan RSK antibody, R&D Systems, 0.5 µg/ml) overnight at 4 °C, and the detection was performed using the BM Chemiluminescence Western blotting kit (Roche Applied Science).

Immunohistochemistry—Decapsulated (treated by 3% sodium hypochlorite solution) embryos and nauplii were fixed in 4% paraformaldehyde and paraaffin-embedded. 5-µm-thick sections were incubated with the anti-phospho-RSK antibody (1:100) or anti-RSK antibody (5 µg/ml) overnight at 4 °C and subsequently incubated with the alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (1:200, Promega). The staining was performed using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution in the dark.

Analysis of Cell Division and Cell Cycle Phase—Cells from embryos and shrimp were collected by crushing, trypsinization (0.25% trypsin, 37 °C), washing, and suspension in ice-
cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.76 mM KH$_2$PO$_4$, pH 7.4) and fixed in 70% ethanol at 4 °C.

For nuclear morphological analysis cells were stained with 4′,6-diamidino-2-phenylindole dilactate (Sigma). For each sample, quintuplicate against three preparations of cell suspension were performed, and more than 20,000 cells were observed.

For flow cytometry analysis, fixed cells were incubated with RNase A (100 μg/ml, Sigma) for 30 min at 37 °C and stained with propidium iodide (50 μg/ml, Sigma) for 30 min at 4 °C. Analysis of DNA content and cell cycle phase was performed using the BD FACSCalibur fluorescence-activated cell analyzer. For each sample duplicates of three preparations of cell suspension were performed, and results were analyzed using Cell Quest™ software version 3.1 (BD Biosciences). All data were given as the mean ± S.E., and all statistical analyses were performed using the one-way ANOVA. The differences were considered significance for $p < 0.01$.

**Double-stranded RNA (dsRNA) Preparation for RNA Interference (RNAi)—** Two oligos (5′-AGCTTGGAATTGTATTCT-CGCTCAATTCCCCTATAGTGATCGATTA-3′ and 5′-TCGATAATACGACTCACTATAGGGGAATTGTGAG-CGGATAACATCCCA-3′, 10 μM each) were mixed together in TN buffer (10 mM Tris, pH 8.0, and 10 mM NaCl), heated at 70 °C for 2 min, and then cooled down slowly to room temperature. The annealed product was subcloned into pET-32a (Novagen) at HindIII and XhoI sites. Subsequently the obtained plasmid (named as pET-T7) was transformed into E. coli DH5α. Nucleotide sequences of the recombinant plasmids expressing dsRNA of _Artemia RSK_ (named as pET-T7-RSK), a 576-bp cDNA fragment in the coding region of the RSK gene was amplified by PCR with primers siF and siR (Table 1), excised, and subcloned into pET-T7 at XbaI and EcoRI sites. All plasmid constructions and propagations were performed in _Escherichia coli_ DH5α. Nucleotide sequences of the recombinant plasmids were confirmed by DNA sequencing. Both the recombinant plasmids of pET-T7-RSK and pET-T7 (as a control) were transformed into _E. coli_ HT115, and the dsRNAs were produced and purified as described by Yodmuang _et al_. (21).

**Embryonic Microinjection—** _Artemia_ embryonic microinjections were performed with decapsulated embryos using the UltraMicroPump II equipped with the Micro4™ MicroSyringe Pump Controller.

For RNAi, each postdiapause embryo was injected with 5 ng of RSK dsRNA or control dsRNA. For kinase inhibition, each 6-h-incubated embryo was injected with 2.3 nl of 1 mM RSK inhibitor SL0101 (Toronto Research Chemicals, Inc.) or an equal volume of Me$_2$SO (control). For antibody neutralization, each 6-h-incubated embryo was injected with 2.3 nl of anti-phospho-RSK antibody or an equal volume of antibody storage buffer (control, 10 mM sodium HEPES, pH 7.5, 150 mM NaCl, 1% Me$_2$SO).
100 μg/ml bovine serum albumin, and 50% glycerol). Injected embryos were incubated in 2.5% artificial seawater at 28 °C with continuous light, and the hatched nauplii were examined and categorized every 12 h.

**RESULTS**

**Identification of RSK Encoding cDNA in Artemia**—To investigate the role of RSK during the embryonic development of *Artemia*, a 2474-bp cDNA encoding RSK was isolated from the postdiapause embryos of *A. parthenogenetica* of Gahai. Sequence analysis reveals the cDNA contains an open reading frame encoding a protein of 714 amino acids, with a predicted mass of 81.3 kDa. The deduced amino acid sequence of this protein is similar to those of other RSK family members. Comparison results show 61% sequence identities with frog, mouse, and human RSK2, 61% with mouse and human RSK1, 60% with mouse and human RSK3, and 46% with mouse and human RSK4. Phylogenetic analysis suggests that *Artemia* RSK can be grouped with the RSK of another arthropod *Drosophila melanogaster* (59% sequence identity).

The deduced amino acid sequence shows that *Artemia* RSK consists of two distinct kinase domains, the N-terminal kinase domain and the C-terminal kinase domain, joined by a linker region. The whole protein contains five RSK typical phosphorylation sites (Ser-218, Thr-359, Ser-363, Ser-380, and Thr-571, shown in Fig. 1) which were identified according to the RSK consensus phosphorylation sites (1, 4). It also displays a special sequence (Leu-Lys-Arg-Arg) in the region from position 673 to 676 that is thought to be necessary for extracellular signal-regulated kinase (ERK).

**RSK Expression and Activation during Embryonic and Larval Developmental Stages**—Northern blot analysis using 20 μg total RNA did not detect the transcript; therefore, semiquantitative reverse transcription-PCR was performed to characterize the expression pattern of RSK gene. The results show that the RSK transcript is universally expressed throughout the whole life cycle of *Artemia* (from diapause or nondiapause embryos through to adults with diapause or nondiapause embryos), and no significant expression fluctuations were observed among different stages (data not shown).

The activation of RSK during the embryonic and larval development processes was analyzed by Western blotting using the phospho-p90RSK (Ser380) antibody, which has been widely used in a cryostat. Sections were incubated with the monoclonal anti-BrdUrd antibody (1:100, Sigma) overnight at 4 °C and subsequently incubated with the anti-mouse IgG-POD antibody (1:250, Roche Applied Science). The staining was performed using the DAB kit (Boster, Wuhan, Hubei, China).
used to detect the endogenous activated p90\(^{\text{RSK}}\) (23–25). The results (Fig. 2) show that RSK is abruptly activated in the 12-h-incubated embryo. Subsequently the phospho-RSK level increases in both the 14-h-incubated embryo and the newly hatched nauplius. During larval development, the phospho-RSK level decreases at the metanauplius stage, and no signal was detected in the postlarva or adults. In contrast, total RSK could be detected at as early a stage as in the diapause embryo, and it subsequently kept relatively constant after developmental resumption (Fig. 2). Immunohistochemical analysis also illustrates that in the emerging embryo (12-h-incubated) most phospho-RSK is located in the endoderm and in the nauplius signals can be detected throughout the whole body covering endoderm, mesoderm, and ectoderm (Fig. 3).

**Association between RSK Activation and Termination of Cell Cycle Arrest during Development of Encysted Embryos**—To explore the connection between RSK activity and mitosis during *Artemia* development, developmental stages of embryos and larvae with the high level phospho-RSK were accurately identified through morphological observation. Fig. 4, a–i show the developmental stages from the postdiapause embryo through to the postlarva. Here we show clearly observable internal differentiation in the 8-h-incubated embryo, prenauplius emergence in the 12-h-incubated embryo, a pear-shaped prenauplius totally escaping from the cyst shell after 14 h of incubation, and the adult form almost achieved at the postlarval stage.

The nuclear morphological observation of cells of each stage (Fig. 4, a′–i′) reveals that there is no observable cell division during preemergence development (postdiapause, 4-h-, 8-h-, and 10-h-incubated), that rare cells divide (at a rate of under 1%) at the beginning of emergence (12-h- and 14-h-incubated), and that the mitosis becomes active after the swimming nauplius formation (when ~3% of cells are mitotic) and becomes further increased (observed at >15%) at the metanauplius stage.

The DNA content and cell cycle phase of each stage was identified by flow cytometry analysis. Five representative stages were analyzed, namely (i) postdiapause, (ii) before emergence (10-hour-incubated), (iii) after emergence (14-h-incubated), (iv) newly hatched nauplius, and (v) postlarval stage. Due to the ubiquitous heteroploidies and aneuploidies in *Artemia* (14, 26), two parameters, the Geo Mean of DNA content with total events and the ratio of M2 events/M1 events (M2 was defined as a G\(_2\)/M region and M1 as a G\(_0\)/G\(_1\) region), were used for the analysis. The results (Fig. 5) show that at the preemergence developmental stages (postdiapause and 10-h-incubated) total DNA content seems to be the highest compared with that of other stages analyzed, most events being in the G\(_2\)/M region. After emergence (14-h-incubated and nauplius), the DNA content is still maintained at a relatively high level; however, more events fall into the G\(_0\)/G\(_1\) region. In the postlarva, total DNA content is much lower, and a large number of events are in the G\(_0\)/G\(_1\) region. These results confirm that RSK activation occurs at the very beginning of emergence and is coupled with the termination of G\(_2\)/M-phase arrest and mitogenesis.

**In Vivo Knockdown of RSK Activity in Artemia**—Embryonic RNAi was performed by dsRNA microinjection, which has been successfully applied in *Artemia* (27, 28).
results show that up to 20% of the surviving individuals injected with RSK dsRNA display severe phenotypes, whereas control groups display no such abnormalities. The majority of these abnormal Artemia, especially at the metanauplius stage, have defects in the developing thoracic and abdominal regions to various degrees (Fig. 6A). In these segments, either partial sections or the entire internal cell layers are much thinner than those of normal individuals, resulting in the formation of areas of distinct gaps between the chitin exoskeleton and the internal tissues. In addition, some other phenotypes (such as the shortening of the antenna, the expod, or the swimming setae) were also observed, although in much smaller numbers (data not shown). The phenotypically affected individuals were unan-
imated in contrast with both the controls and the tested individuals without severe defects. All such affected individuals died before development into the postmetanauplius stage. By real-time PCR and immunohistochemical analysis, it was established that in individuals with an RSK-RNAi phenotype, the RSK transcript level was reduced to 20% approximately, and the phospho-RSK signals were much weaker compared with controls (Fig. 6, B and C). In those unaffected tested individuals, however, the RSK transcript was reduced by only 30–40%.

Likewise, both the RSK specific inhibitor SL0101 and the anti-phospho-RSK antibody were used to directly inhibit RSK activity for the further presentation of RSK activity require-
ment. 15–20% of the survivors injected with either the inhibitor or the antibody presented the same abnormalities as observed in RNAi groups as would be expected (Fig. 7). By the kinase inhibition and the antibody neutralization, tested individuals showed defects earlier compared with those RNAi-treated.

With BrdUrd labeling and detection it was found that tested nauplii showed weaker mitotic activity compared with the controls; at the metanauplius stage mitoses were largely inhibited in the abnormal segments of phenotypically affected individuals (Fig. 8). Mitotic index analysis by nuclear morphological observation also revealed an approximate 50% level of suppression of cell divisions in the affected individuals. These results provide the evidence that RSK activity is indispensable to the mitogenesis, which contributes to the elongation and enlargement of tissues during Artemia development.

**DISCUSSION**

The developmental pattern of Artemia-encysted embryos is unique because of its cell cycle arrest during the preemergence developmental stage from gastrula to pnauplius, which contributes to the capacity to resuspend metabolic activity and, thus, minimizes damage from the various environmental threats (18). However, the mechanism of this process is largely unknown. Our results suggest that RSK plays an important role in the termination of cell cycle arrest and the mitogenesis during the post-embryonic development of Artemia-encysted gastrula.

**The C-terminal Tail of Typical RSKs Lacks in Artemia RSK—**

Recent studies on the structure-function relationship of RSK indicate that the C-terminal tail (Leu-Ala-Gln-Arg-Xaa<sub>5/6</sub>-Ser-Thr-Xaa-leu) containing both the ERK-docking motif and a serine phosphorylation site is considered to be responsible for the ERK docking and kinase activation (29–31). However, some studies have revealed that RSK constructs lacking the C-terminal still show significant constitutive activities in oocytes and cleaving embryos (6, 32, 33). Correspondingly, in vitro phosphorylation of RSK1 at Ser-380 by the PDK1 (3-phosphoinositide-dependent protein kinase-1) was independent of ERK activation (34).

In our study sequence analysis shows that Artemia RSK lacks the typical ERK-docking site in the C-terminal tail even if it displays a sequence necessary to ERK docking in another region. However, Western blotting analysis confirmed that the Ser-380 site of Artemia RSK was phosphorylated at certain...
developmental stages. These findings possibly suggest a special mechanism of in vivo activation of Artemia RSK during post-embryonic development. This is perhaps associated with the role of RSK on mitogenesis that is not yet found in embryos of other species.

Artemia RSK Involvement in Termination of Cell Cycle Arrest and Mitogenesis during Post-embryonic Development—RSKs have been identified with significant roles in cell cycle control, and their functions of mediating meiotic arrest are the subject of most attention (6, 32, 35, 36).

Here our study reveals further aspects of RSK functions. In contrast to former reports that active RSKs induced metaphase arrest in cleaving embryos (6, 32), in this case RSK activation is coupled with the termination of cell cycle arrest and the mitogenesis in encysted embryos. Our results reveal that Artemia RSK activation occurs at the very beginning of emergence simultaneously with mitogenesis, whereas total RSK exists throughout the developmental stages, even with a small quantity being stored in the diapause embryo. In the emerging embryo most phospho-RSK is located in the endoderm where mitotic figures are initially observed, and in the nauplius, signals can be detected throughout the whole body covering endoderm, mesoderm, and ectoderm where imminent mitotic activities will be highly exhibited (Fig. 3).

It is known that the enlargement and elongation of most tissues during Artemia development directly results from great numbers of cell divisions (37). A previous study reported that the delay of cell replication by floxuridine in the instar I nauplius caused a reduction of the cell density in the thoracopod bud, and the larvae formed small segments (38). In this study, in vivo knockdowns of RSK activity consistently induced defective individuals with visible gaps between the chitin exoskeleton and the internal tissues (Figs. 6 and 7). This was ascribed to the thin internal cell layers, and mitoses were indeed largely inhibited in the affected regions (Fig. 8).

Some previous studies have mentioned the interesting G2/M-phase arrest during the preemergence development of Artemia-encysted embryos (14, 16). However, the exact phase of cell cycle arrest is still uncertain. Olson and Clegg (39) considered it a G2-phase arrest according to the heterogeneity in the relative DNA content of individual nuclei in encysted embryos. Nevertheless, we suggest that cells are arrested in the M phase during preemergence development based on the results of flow cytometry analysis representing the arrest in G2/M phase (Fig. 5) and clear chromosome observation (figure not shown). After emergence, mitosis resumes, and a few cells are released from the M-phase arrest. However, the DNA content of those emerging embryos and swimming nauplii remains relatively high. This may be because of the reportedly ubiquitous heteroploidies and polyploidies of cells at these stages (14, 26).

It was reported that a few mitotic figures were observed during the early development of nondiapause embryo in Artemia salina (39, 40). In our experiment RSK was not detected in the nondiapause embryos of A. parthenogenetica of Gahai by Western blotting. This might be due to its low level at this stage. The other possible explanation is that there is another signal pathway for cell cycle regulation in the nondiapause embryonic development of A. parthenogenetica, in that activated RSK is proved to prevent parthenogenetic activation of unfertilized oocytes (6). According to all these results, RSK is probably involved in the termination of M-phase arrest and the promo-
tion of mitogenesis during the post-embryonic development of Artemia-encysted embryos.

Sufficiency of RSK Activity and Mitosis during Artemia Development—During the early development of Artemia-encysted embryos, cell division is totally uncoupled from differentiation and morphogenesis. The mitosis occurs at the beginning of emergence and is highly enhanced during larval development. Although cell division achieves the enlargement and elongation of most tissues, it is believed that even up to the stage II nauplius the development can occur with few mitoses. Olson and Clegg (39) reported that when the newly hatched nauplius was exposed to floxuridine, cell division was largely inhibited, but observable development nevertheless proceeded normally; however, the molting of nauplius at the second or third molt was really arrested or slowed down.

In our RSK knockdown experiments, no phenotypes were observed in the newly hatched nauplius, and most abnormalities appeared at the metanauplius stage in the developing thoracic and abdominal regions. One reason is probably that in the development process of Artemia stronger mitotic activities are required in the thoracic and abdominal segments to ensure the morphogenesis of internal tissues (37), thus, when mitoses are largely inhibited, abnormalities in these regions can be observed more easily and distinctly. Another possibility for no observation of individuals with abnormalities in the whole body or the head region is that defects of such degrees or in such important parts (containing the brain) are lethal, and all the affected ones die before being observed.

These facts illustrate that RSK activity is indispensable in the post-embryonic and early larval development of Artemia. This is because of its significant function on the termination of cell cycle arrest and the promotion of mitogenesis that is crucial to the larval development at later stages, although few mitoses are needed in the newly hatched nauplius.

Acknowledgments—We thank Dr. Chen Luo and Dr. Yong Wang of Zhejiang University for technical assistance. We also thank Chris Wood of Zhejiang University for critical reading of this manuscript. E. coli HT115 was kindly provided by the Caenorhabditis Genetics Center, which is funded by the National Center for Research Resources.

REFERENCES
1. Haug, C., and Frodin, M. (2006) J. Cell Sci. 119, 3021–3023
2. Jones, S. W., Erikson, E., Blenis, J., Maller, J. L., and Erikson, R. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3377–3381
3. Dalby, K. N., Morrice, N., Caudwell, F. B., Avruch, J., and Cohen, P. (1998) J. Biol. Chem. 273, 1496–1505
4. Roux, P. P., and Blenis, J. (2004) Microbiol. Mol. Biol. Rev. 68, 320–344
5. Wassarman, D. A., Solomon, N. M., and Rubin, G. M. (1994) Gene (Amst.) 144, 309–310
6. Mori, M., Hara, M., Tachibana, K., and Kishimoto, T. (2006) Development 133, 1823–1830
7. Fujita, N., Sato, S., and Tsuruo, T. (2003) J. Biol. Chem. 278, 49254–49260
8. Smith, J. A., Poteet-Smith, C. E., Xu, Y., Errington, T. M., Hecht, S. M., and Lannigan, D. A. (2005) Cancer Res. 65, 1027–1034
9. Godeny, M. D., and Sayeski, P. P. (2006) Am. J. Physiol. 291, C1308–C1317
10. Slegers, H. (1991) in Artemia Biology (Browne, R. A., Sorgeloos, P., and Trotman, C. N. A., eds) pp. 37–73, CRC Press, Inc., Boca Raton, FL
11. Clegg, J. S. (1978) in Dry Biological Systems (Crowe, J. H., and Clegg, J. S., eds) pp. 117–153, Academic Press, Inc., New York
12. Drinkwater, L. E., and Clegg, J. S. (1991) in Artemia Biology (Browne, R. A., Sorgeloos, P., and Trotman C. N. A., eds) pp. 93–117, CRC Press, Inc., Boca Raton, FL
13. Nakamichi, Y. H., Iwasaki, T., Okigaki, T., and Kato, H. (1962) Annot. Zool. Jpn. 35, 223–228
14. Nakamichi, Y. H., Okigaki, T., Kato, H., and Iwasaki, T. (1963) Proc. Jpn. Acad. 39, 306–309
15. Olson, C. S., and Clegg, J. S. (1976) Experientia (Basel) 32, 864–865
16. Fin amore, J. J., and Clegg, J. S. (1969) in The Cell Cycle: Gene-Enzyme Interactions (Padilla, G. M., Whitson, G. L., and Cameron, I. L., eds) pp. 249–278, Academic Press, Inc., New York
17. Hentschel, C. C., and Tata, J. R. (1976) Trends Biochem. Sci. 1, 97–100
18. Clegg, J. S., and Trotman C. N. A. (2002) in Artemia: Basic and Applied Biology (Abatzopoulos, T. J., Beardsmore, J. A., Clegg, J. S., and Sorgeloos, P., eds) pp. 129–197, Kluwer Academic Publishers, Dordrecht, Netherlands
19. Dai, Z. M., Zhu, X. J., Chen, Q., and Yang, W. J. (2007) J. Biotechnol. 128, 435–443
20. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
21. Yodnuang, S., Tirarophorn, W., Roshhorn, Y., Chinnirunwong, W., and Panyim, S. (2006) Biochem. Biophys. Res. Commun. 341, 351–356
22. Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408
23. Majka, M., Janowska–Wieczorek, A., Ratajczak, J., Kowalska, M. A., Vilaire, G., Pan, Z. K., Honczarenko, M., Marquez, L. A., Ponez, M., and Ratajczak, M. Z. (2000) Blood 96, 4142–4151
24. Tomas-Zuber, M., Mary, J. L., and Lesslauer, W. (2000) J. Biol. Chem. 275, 23549–23558
25. Hans, A., Syan, S., Crosisio, C., Sassone-Corsi, P., Brahic, M., and Gonzalez-Dunia, D. (2001) J. Biol. Chem. 276, 7258–7265
26. Gajardo, G., Abatzopoulos, T. J., Kappas, I., and Beardsmore, J. A. (2002) in Artemia: Basic and Applied Biology (Abatzopoulos, T. J., Beardsmore, J. A., Clegg, J. S., and Sorgeloos, P., eds) pp. 225–250, Kluwer Academic Publishers, Dordrecht, Netherlands
27. Copf, T., Schroder, R., and Averof, M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17711–17715
28. Copf, T., Rabet, N., and Averof, M. (2006) Dev. Biol. 298, 87–94
29. Bjorbaek, C., Zhao, Y., and Moller, D. E. (1995) J. Biol. Chem. 270, 18848–18852
30. Fisher, T. L., and Blenis, J. (1996) Mol. Cell. Biol. 16, 1212–1219
31. Roux, P. P., Richards, S. A., and Blenis, J. (2003) Mol. Cell. Biol. 23, 4796–4804
32. Gross, S. D., Schwab, M. S., Lewellyn, A. L., and Maller, J. L. (1999) Science 286, 1365–1367
33. Gross, S. D., Lewellyn, A. L., and Maller, J. L. (2001) J. Biol. Chem. 276, 46099–46103
34. Fordin, M., and Gammeltoft, S. (1999) Mol. Cell. Endocrinol. 151, 65–77
35. Palmer, A., Gavvin, A. C., and Nebreda, A. R. (1998) EMBO J. 17, 5037–5047
36. Bhatt, R. R., and Ferrell, J. E., Jr. (1999) Science 286, 1362–1365
37. Criel, G. R. I., and MacRae, T. H. (2002) in Artemia: Basic and Applied Biology (Abatzopoulos, T. J., Beardsmore, J. A., Clegg, J. S., and Sorgeloos, P., eds) pp. 39–128, Kluwer Academic Publishers, Dordrecht, Netherlands
38. Freeman, J. A., Cheshire, L. B., and MacRae, T. H. (1992) Dev. Biol. 152, 279–292
39. Olson, C. S., and Clegg, J. S. (1978) Roux’s Arch. Dev. Biol. 184, 1–13
40. Benesch, R. (1969) Zool. Jordbrukstek. Anat. 86, 307–458