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Possible involvement of insulin-like growth factor 2 mRNA-binding protein 3 in zebrafish oocyte maturation as a novel *cyclin B1* mRNA-binding protein that represses the translation in immature oocytes

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ABSTRACT

In immature zebrafish oocytes, dormant *cyclin B1* mRNAs localize to the animal polar cytoplasm as aggregates. After hormonal stimulation, *cyclin B1* mRNAs are dispersed and translationally activated, which are necessary and sufficient for the induction of zebrafish oocyte maturation. Besides cytoplasmic polyadenylation element-binding protein (CPEB) and *cis*-acting elements in the 3′ untranslated region (UTR), Pumilio1 and a *cis*-acting element in the coding region of *cyclin B1* mRNA are important for the subcellular localization and timing of translational activation of the mRNA. However, mechanisms underlying the spatio-temporal control of *cyclin B1* mRNA translation during oocyte maturation are not fully understood. We report that insulin-like growth factor 2 mRNA-binding protein 3 (IMP3), which was initially described as a protein bound to *Vg1* mRNA localized to the vegetal pole of *Xenopus* oocytes, binds to the 3′ UTR of *cyclin B1* mRNA that localizes to the animal pole of zebrafish oocytes. IMP3 and *cyclin B1* mRNA co-localize to the animal polar cytoplasm of immature oocytes, but in mature oocytes, IMP3 dissociates from the mRNA despite the fact that its protein content and phosphorylation state are unchanged during oocyte maturation. IMP3 interacts with Pumilio1 and CPEB in an mRNA-dependent manner in immature oocytes but not in mature oocytes. Overexpression of IMP3 and injection of anti-IMP3 antibody delayed the progression of oocyte maturation. On the basis of these results, we propose that IMP3 represses the translation of *cyclin B1* mRNA in immature zebrafish oocytes and that its release from the mRNA triggers the translational activation.

1. Introduction

Immature oocytes become mature (fertilizable) by the activity of maturation-promoting factor (MPF), a protein kinase consisting of the catalytic subunit Cdc2 and regulatory subunit *cyclin B1*. Since *cyclin B1* is absent in immature oocytes of fish and amphibians except *Xenopus laevis*, its *de novo* synthesis is essential for the initiation of oocyte maturation in these species [1–3]. Protein synthesis during oocyte maturation chiefly depends on the translational activation of maternal mRNAs, which are synthesized during oocyte growth and stored in dormant forms in the oocytes until activated at timings specific to each mRNA. The translational activation of maternal mRNAs, including *cyclin B1* mRNA, is triggered by cytoplasmic polyadenylation, which is mainly regulated by the cytoplasmic polyadenylation element-binding protein (CPEB) that resides in the 3′ untranslated region (UTR) [4]. Besides CPEB, however, other RNA-binding proteins specific to each mRNA are required for the accurate timings of translation. For example, the strict temporal order of *mos*, *cyclin B1* and *wee1* mRNA translation, which is important to ensure the normal progression of oocyte maturation, is regulated by CPEB in cooperation with certain partners, Musashi for *mos* [5], Pumilio1 for *cyclin B1* [6] and Zar2 for *wee1* [7].

Fish oocytes are characterized by the micropyle, a sperm entry hole on the egg chorion at the animal pole (Supplementary Fig. 1A), while amphibian (*Xenopus*) oocytes lack it. In zebrafish, dormant *cyclin B1* mRNAs aggregate and localize to the animal polar cytoplasm beneath the micropyle (Supplementary Fig. 1B), whereas similar aggregation and localization have not been reported for *Xenopus cyclin B1* mRNA. After hormonal stimulation, *cyclin B1* mRNAs disperse, leading to translational activation of the mRNA, an event necessary and sufficient for the induction of oocyte

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maturation in zebrafish [8]. We have previously shown that Pumilio1 bound to the 3′ UTR and an unidentified protein(s) bound to the coding region of *cyclin B1* mRNA are required for the correct localization and temporal regulation of translational activation of the mRNA [9–11]; however, we are still far from a comprehensive understanding of mechanisms that ensure the spatio-temporally regulated translational activation of *cyclin B1* mRNA during zebrafish oocyte maturation. Here, we report that IMP3 is a novel *cyclin B1* mRNA-binding protein in immature zebrafish oocytes and suggest that it represses the translation of *cyclin B1* mRNA in immature oocytes.

2. Materials and methods

2.1. Oocyte culture and extraction

All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University (No. 13-0099). Full-grown oocytes were manually isolated from ovaries and placed in zebrafish Ringer's solution [8]. Maturation was induced by incubating the oocytes in culture medium (90% Leibovitz's L-15, 0.5% bovine serum albumin (BSA), 100 μg/ml gentamycin; pH 9.0) containing 1 μg/ml 17α,20β-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish. The progression of maturation was assessed by the occurrence of germinal vesicle breakdown (GVBD). At appropriate times after hormonal stimulation, oocytes were homogenized with a pestle to obtain oocyte extracts (1 μl/oocyte), as described previously [9].

2.2. Production of recombinant proteins and antibodies

To produce a full-length IMP3 with a Flag tag at the N terminus (Flag-IMP3), an ORF of zebrafish IMP3 (NM_131491.2) was amplified with a primer set which introduces *Cla*I and *Xho*I sites (5′-ATCGATatgaataagctgtacatcgggaa-3′ and 5′-CTCGAGtttcctcctgg cgactg-3′). The PCR product was cloned into pGEM-T easy vector (Promega), digested with *Cla*I and *Xho*I, and ligated into pCS2-Flag-N.

To produce a truncated IMP3 tagged with a glutathione-S-transferase (GST) at the N terminus (GST-IMP3) or a polyhistidine (His) at the C terminus (IMP3-His), a cDNA fragment of 589–1227 of zebrafish IMP3 was amplified with a primer set (5′-caccctac gcttgctggtaccg-3′ and 5′-ttccgactccatctgcg-3′). The PCR product was cloned into pENTR/D-TOPO vector (Invitrogen) and the resulting plasmid was recombined with the destination vectors pDEST15 for GST-IMP3 and pET161-DEST for IMP3-His, using the Gateway cloning system (Invitrogen).

GST-IMP3 and IMP3-His expressed in *Escherichia coli* BL21 (DE3) were purified by SDS–PAGE followed by electro-elution in Tris–glycine buffer without SDS, and they were injected into mice to produce polyclonal antibodies. IMP3-His was electroblotted onto an immobilon membrane and used to affinity purify the antibodies.

Using a full-length cDNA clone (AB044534) in pBluescript SK, an ORF of goldfish CPEB was amplified with a primer that introduces a *Bgl*II site just before the first ATG codon (5′-gaAGATCTatggcgtttt ctctgagc-3′) and the T7 primer. The resulting cDNA was digested with *Bgl*II and *Eco*RV and ligated into *Bam*HI-*Eco*RV-cut pET3a vector (Novagen). Monoclonal antibodies were raised against *E. coli*-produced proteins. Two monoclonal antibodies (GFCPEB 3D3 and 9G3) recognize zebrafish CPEB (Supplementary Fig. 2A).

Digoxigenin (DIG) was coupled with BSA or keyhole limpet hemocyanin using DIG-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (Roche) and was injected into guinea pigs and rabbits to produce anti-DIG antibodies. Since an antiserum from the guinea pig injected with DIG-conjugated BSA showed high specificity to DIG, it was used in combination with Alexa 488-labeled secondary antibody to detect DIG-labeled *cyclin B1* probes under a fluorescent microscope for section *in situ* hybridization analysis, as described below.

2.3. Immunoblotting and immunoprecipitation

Immunoblotting was performed as described previously [12], using anti-IMP3, anti-Pumilio1 (Pum2A5) [13], anti-CPEB (GFCPEB 3D3 or 9G3), anti-Cdc2 (MC2-21) [1], anti-phospho-MAP kinase (#9101; Cell Signaling Technology), anti-γ-tubulin (T6557; Sigma–Aldrich) or anti-Flag (F1804; Sigma–Aldrich) antibodies. The interaction of IMP3 with Pumilio1 or CPEB was examined by immunoprecipitation, as described previously [12]. To detect phosphorylation of IMP3, immature and mature oocyte extracts were immunoprecipitated with anti-IMP3 antibody. The resulting precipitates were treated with λ-phosphatase (200 U, New England Biolabs) for 1 h at 30 °C, separated by SDS–polyacrylamide gel containing 20 μM Phos-tag acrylamide (NARD institute) and 40 μM $MnCl_2$, and immunoblotted with anti-IMP3 antibody.

2.4. Sucrose density gradient ultracentrifugation

Zebrafish ovaries were homogenized in 500 μl of gradient buffer (80 mM KCl, 20 mM NaCl, 10 mM Tris–HCl, 20 mM EDTA; pH 7.4) and centrifuged at 5000 rpm for 5 min at 4 °C. The supernatant was loaded onto 4 ml of 0.1–1.9 M sucrose density gradient prepared in gradient buffer. After centrifugation at 35,000 rpm in a Hitachi S52ST rotor for 5 h at 4 °C, 450-μl fractions were collected from the bottom of the tube. *Cyclin B1* mRNA contents in each fraction were analyzed by northern blotting, according to the procedure reported previously [8]. The fractions were also analyzed by immunoblotting to detect Cdc2, Pumilio1, CPEB and IMP3 and by SDS–PAGE followed by silver staining to detect total protein.

2.5. UV cross-linking assay

mRNAs encoding Flag-IMP3 and Flag-GST were synthesized with an mMESSAGE mMACHINE SP6 kit (Ambion), and the resulting mRNAs (2 μg) were translated in 50 μl of rabbit reticulocyte lysate (Promega). Flag-IMP3 and Flag-GST were purified by immunoprecipitation with anti-Flag antibody. The 3′ UTRs of zebrafish *cyclin B1* and β-*globin* mRNAs were amplified by RT-PCR with the following primer sets: for *cyclin B1*, 5′-ttggggttatgctg-3′ and 5′-aaaactttaaaaagtttatttgaa-3′; for β-*globin*, 5′-agtctcatcgccaatgaacg-3′ and 5′-gcttttaacattattttattgat-3′. The resulting cDNAs were cloned into pGEM T-easy vector (Promega), and DIG-labeled RNA probes were synthesized with T7 RNA polymerase and a DIG RNA labeling mix (Roche). Cross-linking was performed as described previously [9].

2.6. RT-PCR analysis following immunoprecipitation (IP/RT-PCR)

After extraction of RNA from anti-IMP3 immunoprecipitates with Isogen (Nippon Gene), cDNA was synthesized with a Super-Script III First-Strand Synthesis System for RT-PCR (Invitrogen). Subsequent PCRs were performed with Taq DNA Polymerase (Ampliqon) and the following primer sets: for *cyclin B1*, 5′-gagggcc tttctaagcatctggctgtg-3′ and 5′-ttatttgaattcaaatgtacaaacttgc-3′; for *mos*, 5′-tataacctgcgccctttgaccagc-3′ and 5′-acatttttgcataaaaaatttagc ttcac-3′; for *wee1*, 5′-ttttatccatccaagcaagcgagc-3′ and 5′-tttacaaacaa agagttaacaagacc-3′; for β-*actin*, 5′-ggtagttgtctaacaggggagagc-3′ and 5′-gttgacttgtcagtgtacagaga-3′.