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学位論文題目	Cyclin A and B in Goldfish(Carassius auratus): Their Roles and Mechanisms of Synthesis during Hormone-Induced Oocyte Maturation
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論文内容の要旨

Oocyte maturation is a prerequisite for successful fertilization. This process in fish oocytes has been reported to be regulated by three major mediators: gonadotropin, maturation-inducing hormone ($17\alpha, 20\beta$ -dihydroxy-4-pregnen-3-one, $17\alpha, 20\beta$ -DP, in fish) and maturation-promoting factor (MPF, a complex of cdc2 kinase and cyclin B). Cyclins have been reported to regulate the kinase activity of cdc2, leading to the activation and inactivation of MPF. The present study investigates the roles of cyclin A and B and the mechanisms of translational activation of maternal cyclin B mRNA during $17\alpha, 20\beta$ -DP-induced oocyte maturation in goldfish (*Carassius auratus*).

Western blot analysis was used to examine changes in the protein levels of cdc2 kinase and cyclin B during goldfish oocyte maturation induced *in vitro* with $17\alpha, 20\beta$ -DP. Immature oocytes contained a 35-kDa cdc2. In addition to this protein, a 34-kDa cdc2 was detected in mature oocytes. The purified MPF contained the 34-kDa cdc2, but not 35-kDa cdc2. Thus, it is concluded that the 34- and 35-kDa cdc2 proteins are active and inactive forms, respectively. The 34-kDa active cdc2 appeared in accordance with the onset of germinal vesicle breakdown (GVBD). Cyclin B was absent in immature oocyte extracts and appeared when oocytes underwent GVBD, coinciding with the appearance of the 34-kDa active cdc2. Precipitation experiments with p13^{sup} and anti-cyclin B antibody revealed that cyclin B that appeared during oocyte maturation formed a complex with cdc2, as soon as it appeared. It is most likely that the 35-kDa inactive cdc2 preexisting in immature oocytes forms a complex with *de novo* synthesized cyclin B at first, then is immediately converted into the 34-kDa active form, which triggers all changes that accompany oocyte maturation, such as GVBD, chromosome condensation and spindle formation. Introduction of *E. coli*-produced cyclin B into immature oocytes using microinjection induced oocyte maturation under condition of inhibited protein synthesis. These results strongly suggest that MPF activation in fish oocytes is induced by complex formation with preexisting cdc2 kinase and newly synthesized cyclin B during oocyte maturation, a situation differing from that in *Xenopus* and starfish, in which the cdc2 kinase-cyclin B complex is already present in immature oocytes.

Both in *Xenopus* and fish, unfertilized mature oocytes are arrested at the second meiotic metaphase until fertilized. In contrast to *Xenopus*, an inhibition of protein synthesis in unfertilized mature goldfish oocytes with cycloheximide caused a 30-50% decrease in the cdc2 kinase activity/cyclin B protein levels and an exit from meiotic metaphase-arrest. As compared with that occurring upon normal activation, the induced-decrease in the MPF activity was

partial, and the cell cycle of the cycloheximide-treated oocytes was arrested again at the second meiotic anaphase. These results show that cdc2 kinase activity, cyclin B protein level, and cell cycle progression are closely linked. Furthermore, it is suggested that in addition to a difference in the mechanisms of MPF activation, the mechanisms of maintaining MPF activity in unfertilized mature goldfish oocytes differ from those in mature *Xenopus* oocytes.

Although cyclin A is thought to be involved in the regulation of both S and M phase in eukaryotic cell cycle, its exact role in the cell cycle, especially in the meiotic cycle (oocyte maturation) is uncertain. To investigate the role of cyclin A in oocyte maturation, a goldfish cyclin A cDNA was cloned and antibodies against its product were produced. Unlike goldfish cyclin B that is absent in immature oocytes, cyclin A was already present in immature oocytes and its protein level was not remarkably changed during oocyte maturation. These observations differ from those of *Xenopus* oocytes, showing an undetectable amount of cyclin A and a large amount of stockpiled cyclin B at the onset of oocyte maturation. Thus, the behavior of goldfish cyclin A resembles that of *Xenopus* cyclin B, whereas that of goldfish cyclin B resembles *Xenopus* cyclin A. In the goldfish oocyte system, cyclin A binds to cdc2, but not cdk2, and that it activates cdc2 both *in vivo* and *in vitro*, raising the possibility that cyclin A plays a role in oocyte maturation. Changes in cyclin A-cdc2 and cyclin B-cdc2 kinase activity during oocyte maturation were also examined. Cyclin B-cdc2 kinase activity increases according to the occurrence of GVBD. Although the timing of the activation of the cyclin B-cdc2 and cyclin A-cdc2 complexes is almost the same, the rapid increase in cyclin A-cdc2 kinase activity occurs only after the completion of GVBD. It is possible that cyclin A-cdc2 kinase may play an important role in steps after GVBD; for example, the kinase may help the rapid activation of cyclin B-cdc2 kinase at meiosis I to II transition or play a part in the maintenance of high cyclin B-cdc2 kinase activity in mature unfertilized oocytes. It is concluded from these results that cyclin B-cdc2 kinase, but not cyclin A-cdc2 kinase, is important for oocyte maturation (especially GVBD).

In the preceding sections, it was demonstrated that in goldfish oocytes *de novo* synthesis of cyclin B protein is required for the activation of MPF (cyclin B-cdc2 complex) during oocyte maturation. The next series of experiments were designed to investigate the mechanism of $17\alpha, 20\beta$ -DP-induced cyclin B synthesis. It was found that $17\alpha, 20\beta$ -DP-induced oocyte maturation was inhibited by a protein synthesis inhibitor (cycloheximide), but not by an RNA synthesis inhibitor (actinomycin D). Northern blot analysis showed that cyclin B mRNA is present in both immature and mature oocytes with no significant difference between them. Taken together, these results suggest that the

synthesis of cyclin B protein is regulated at the translational level. Since it has long been recognized that the translational activity of maternal mRNAs generally correlates with changes in polyadenylation, the involvement of polyadenylation in cyclin B mRNA translation was examined. Examination of the 3' UTR of goldfish cyclin B mRNA revealed that it possesses a conserved sequence AAUAAA with four copies of cytoplasmic polyadenylation element (CPE, consensus U₄-₆A₁-₂U) motifs which are the *cis*-acting sequence that specifies cytoplasmic polyadenylation. A PCR poly (A) test revealed that poly (A) elongation occurs in goldfish cyclin B mRNA during oocyte maturation. However, it is noteworthy that this poly (A) addition occurred in oocytes which underwent GVBD, that is, after the appearance of cyclin B protein in oocytes, probably after MPF activation. Thus, it is concluded that cyclin B mRNA polyadenylation is not the major mechanism which is responsible for the initiation of cyclin B mRNA translation in goldfish oocytes.

It has also been suggested that some RNA-binding proteins mediate translational process of stored maternal mRNAs. When poly (A)⁺RNA from goldfish immature oocytes was mixed with the reticulocyte lysate system, cyclin B could be synthesized, suggesting the translational ability of goldfish maternal cyclin B mRNA. Furthermore, the efficiency of cyclin B translation was much lower with extracts from immature oocytes than with those from mature oocytes. Thus, it is most likely that the initiation of cyclin B synthesis in goldfish oocytes is regulated by a translational inhibitory factor (RNA-binding protein). In this study, a cDNA clone encoding a goldfish Y box protein, which is known to sequester mRNA from translation in *Xenopus* oocytes, was isolated and partially characterized. The identification of the inhibitory factor and clarification of its role should elucidate the translational regulation of cyclin B synthesis during hormone-induced oocyte maturation in goldfish. Thus, the goldfish oocyte will provide a valuable model to gain better understanding of a basic mechanism for translational regulation of gene expression which has currently been accumulating as a primary regulatory mechanism in eukaryotic systems.

審査結果の要旨

卵成熟促進因子 (maturation-promoting factor, MPF) は動物卵に減数分裂を再開させ受精能を獲得させる成熟促進因子であるばかりでなく、細胞分裂一般においてもG2期からM期へ移行させるM期促進因子でもある。MPFは触媒ユニットのcdc2キナーゼと調節ユニットのサイクリンからなることが知られているが、申請者の勝 義直は、キンギョ (*Carassius auratus*) の卵成熟におけるサイクリンAとBの役割を明らかにするとともに、卵成熟誘起ホルモン (魚類では $17\alpha, 20\beta$ -ジヒドロキシ-4-フルゲネン-3-オン, $17\alpha, 20\beta$ -DPと略) によるサイクリンBの合成機構を調べた。

$17\alpha, 20\beta$ -DPにより誘起された卵成熟過程におけるcdc2とサイクリンBの挙動を調べ、未成熟卵には35-kDaのcdc2が含まれ、成熟卵にはこれに加え34-kDaの活性型cdc2が存在することを示した。一方、サイクリンBは未成熟卵には存在せず、卵核胞崩壊直前に活性型cdc2が出現する時期に一致してはじめて現れる。さらに、サイクリンBは出現直後直ちにcdc2と複合体を形成すること、またキンギョサイクリンBを未成熟卵に微小注射すると卵成熟が誘起されることを明らかにした。

次に、キンギョサイクリンAのcDNAクローニングと特異的抗体の作成に成功し、これらをプローブとして卵成熟時におけるサイクリンAの役割を調べた。サイクリンAタンパク質は未成熟卵中でもすでに存在し、 $17\alpha, 20\beta$ -DP処理の後でも量的変動は認められないことを示した。また、キナーゼ活性は卵核胞崩壊後に急激に上昇し、成熟卵ではcdc2と複合体を形成する。さらに、サイクリンAmRNAの微小注射では卵成熟は誘起されないことを示した。

以上の結果より、魚類の卵成熟は卵成熟誘起ホルモンによるサイクリンBの合成が引金となり起こることが判明したので、次にその合成開始機構を解析した。 $17\alpha, 20\beta$ -DPによる卵成熟はシクロヘキシミドで阻害されるが、アクチノマイシンDでは阻害されないこと、未成熟卵ですでにサイクリンBmRNAは存在すること、未成熟卵のRNAからサイクリンBタンパク質の合成が誘導できることから、 $17\alpha, 20\beta$ -DPによるサイクリンBの合成開始は翻訳レベルで制御されていることが明らかになった。さらに、このサイクリンBmRNAの翻訳が促進される過程に、翻訳抑制因子の不活性化、ポリアデニル化等が関わる可能性を示唆した。

本研究は、卵成熟がmRNAの翻訳開始に始まるサイクリンBの de novo の合成によることを示した最初の研究例であり、動物卵の成熟機構の研究のみならず細胞周期一般の調節機構の研究に大きく貢献すると判断され、審査委員会は本博士論文に対して、合格の判定を下した。

また、学位論文として提出された研究結果について口頭発表させた後、審査委員が論文内容について試問した。さらに、申請者の関連研究分野の一般知識およびその背景となる基礎的知識についても口頭試問により審査した。これらの試問に対する申請者の応答はいずれも的確であった。また、提出された学位論文は英文で書かれており、英語の能力についても適性であると考えられた。さらに、論文の一部はすでに発生生物学分野の国際誌である *Developmental Biology* に掲載されている。これらの結果をもとに、審査委員会は申請者の持つ研究能力および学力は学位取得に値するものと判定した。