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Localization and enzymatic activity profiles of the proteases responsible for tachykinin-directed oocyte growth in the protochordate, *Ciona intestinalis*

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ABSTRACT

We previously substantiated that Ci-TK, a tachykinin of the protochordate, *Ciona intestinalis* (Ci), triggered oocyte growth from the vitellogenic stage (stage II) to the post-vitellogenic stage (stage III) via up-regulation of the gene expression and enzymatic activity of the proteases: cathepsin D, carboxypeptidase B1, and chymotrypsin. In the present study, we have elucidated the localization, gene expression and activation profile of these proteases. In situ hybridization showed that the Ci-cathepsin D mRNA was present exclusively in test cells of the stage II oocytes, whereas the Ci-carboxypeptidase B1 and Ci-chymotrypsin mRNAs were detected in follicular cells of the stage II and stage III oocytes. Double-immunostaining demonstrated that the immunoreactivity of Ci-cathepsin D was largely colocalized with that of the receptor of Ci-TK, Ci-TK-R, in test cells of the stage II oocytes. Ci-cathepsin D gene expression was detected at 2 h after treatment with Ci-TK, and elevated for up to 5 h, and then slightly decreased. Gene expression of Ci-carboxypeptidase B1 and Ci-chymotrypsin was observed at 5 h after treatment with Ci-TK, and then decreased. The enzymatic activities of Ci-cathepsin D, Ci-carboxypeptidase B1, and Ci-chymotrypsin showed similar alterations with 1-h lags. These gene expression and protease activity profiles verified that Ci-cathepsin D is initially activated, which is followed by the activation of Ci-carboxypeptidase B1 and Ci-chymotrypsin. Collectively, the present data suggest that Ci-TK directly induces Ci-cahtepsin D in test cells expressing Ci-TK receptor, leading to the secondary activation of Ci-chymotrypsin and Ci-carboxypeptidase B1 in the follicle in the tachykininergic oocyte growth pathway.

1. Introduction

Tachykinins (TKs) are multifunctional vertebrate brain/gut peptides [14,28,36]. The mammalian TK family consists of four major peptides: substance P (SP), neurokinin A (NKA), neurokinin B (NKB), and hemokinin-1/endokinins (HK-1/EKs). TKs are known to participate in smooth muscle contraction, vasodilation, nociception, inflammation, neurodegeneration, and neuroprotection in a neuropeptidergic or endocrine fashion [2,14–18,31,37], and thus, both central and peripheral tachykininergic systems have become attractive as targets for development of various clinical agents [7,19,26,31]. The tissue-distribution of TKs and TK receptors in the genital organs was detected by reverse transcription (RT)-PCR, immunostaining or radioimmunoassay [5,14,19–24,28,31]. Several recent studies also reported the physiological or pathological effects regulated by TKs in reproductive tracts including sperm motility induced by SP [28], uterine contractions stimulated by SP, NKA, and NKB [20–22,24], the elevation of circulating NKB in pre-eclampsia or pregnancy via enhanced expression of the TAC3 gene in the placenta [14,18], and the stimulation of gonadotropin-releasing hormone release by NKB in concert with kisspeptin in the hypothalamus [27,36]. These findings suggest the multifunctionality of TKs in genital organs. Nevertheless, the biological roles of TKs in the ovary remained to be elucidated, although the expression of NK1 to NK3 was detected in the mammalian ovary by RT-PCR [8].

In the previous study, we showed a novel biological function of TKs as an inducible factor for oocyte growth using the protochordate (ascidian) *Ciona intestinalis* [3]. Ci-TK, the authentic TK characterized from *C. intestinalis* [32], activated the gene expression and resulting enzymatic activities of the proteases, cathepsin D, chymotrypsin, and carboxypeptidase B1 in *Ciona* vitellogenic (stage II) oocytes, which resulted in a progression of growth from the vitellogenic stage to the postvitellogenic stage (stage III) [3]. Furthermore, the Ci-TK-induced oocyte growth was blocked by either a TK antagonist or by a protease inhibitor for each of cathepsin D, chymotrypsin, or carboxypeptidase B1 [3]. These results lead to the conclusion that Ci-TK enhances vitellogenic oocyte growth via upregulation of the gene expression and enzymatic activities

Abbreviations: Ci, *Ciona intestinalis*; TK, tachykinin; Ci-TK-R, Ci-TK receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GVBD, germinal vesicle breakdown; EK, endokinin; HK, hemokinin; NKA, neurokinin A; NKB, neurokinin B; SP, substance P; RT, reverse transcription.

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of cathepsin D, chymotrypsin, or carboxypeptidase B1 [3]. This is the first identification of the biological roles of TKs in the ovary as well as oocyte growth. However, the molecular mechanism by which these proteases induce growth of *Ciona* oocytes remains unknown, although cathepsin D, carboxypeptidase B1, and chymotrypsin were found to be responsible for multiple biological events in the growth of oocytes and follicle cells in various animal species, such as the proteolytic processing of vitellogenin into yolk protein by cathepsin D in vertebrates [6], the proteolytic production of several component proteins for zona pellucida by carboxypeptidase B1 in mammalian oocytes at an early growth stage [11], and unknown mechanism of chymotrypsin activity in insects, echinoderms, and non-*Ciona* tunicates [9,30,34,35]. To address these issues, we investigated the localization of the transcripts and proteins of the three ovarian proteases and the alterations in gene expression and protease activity in the presence of Ci-TK. In the present study, we show the novel molecular mechanism of the growth of stage II oocytes in which Ci-cathepsin D, coexpressed with Ci-TK-R in test cells of stage II oocytes, is initially and directly up-regulated by Ci-TK, and the activation of the other proteases is subsequently induced in follicular cells. To the best of our knowledge, this is the first report on the localization and time-course of gene expression and enzymatic activity of proteases regulating oocyte growth.

2. Materials and methods

2.1. Animals

Adults of *C. intestinalis* were cultivated and collected at the Maizuru Fisheries Reseach Station of Kyoto University, and maintained in seawater at 18 °C.

2.2. Immunohistochemistry

The *Ciona* ovaries were fixed overnight at 4 °C in Bouin fluid, embedded in paraffin, and cut into 15-μm sections. Anti-Ci-Cathepsin D rabbit antiserum and anti-Ci-TK-R chicken antiserum were ordered from Operon Biotechnologies Inc. (Tokyo, Japan). As the peptide antigens, we selected the sequences of YALNNDWYFGKAFC and KYTQHWARQT corresponding to Ci-TK-R 99–112 aa and Ci-Cathepsin D 41–50 aa, respectively. Immunostaining using Ci-Cathepsin D and Ci-TK-R antisera diluted to 1:1000 was performed as previously described [34]. The specificity of the Ci-Cathepsin D antibody was confirmed by Western blotting on membrane proteins prepared from the *Ciona* ovaries (data not shown). The specificity of the Ci-TK-R was confirmed in the previous study [3]. The immunoreactivity was visualized with an indirect immunofluorescence technique using 1:1000 diluted secondary antibody alexa 488 goat anti-rabbit IgG and alexa 568 goat anti-chicken IgG (Molecular Probes Inc., Eugene, OR, USA). Coverslips were mounted in Fluorosafe mounting medium (Calbiochem) and viewed using a Nikon Eclipse TE2000-S photomicroscope equipped with epifluorescence. *Ciona* oocytes are known to mature through four characteristic stages [4,25]: small pre-vitellogenic oocytes (stage I), vitellogenic oocytes (stage II), and postvitellogenic oocytes (stage III), and germinal vesicle breakdown (GVBD) mature oocytes (stage IV). Each growth stage of *Ciona* oocytes was classified on the basis of morphological characteristics such as the structure of follicular cells and oocyte size [4,25]. The data were confirmed by three experiments using independent ovary samples.

2.3. In situ hybridization

The open reading frames of the Ci-carboxypeptidase B1, Ci-chymotrypsin and Ci-cathepsin D cDNA fragments [3] were inserted into the pCRII-TOPO dual promoter vector (Invitrogen, San Diego, CA, USA), respectively. These plasmids were linearized, and supplied to the synthesis of digoxigenin-labeled RNA probes using a digoxigenin RNA labeling kit (Roche Applied Science). The *Ciona* ovary was dissected and fixed in Bouin's fluid at 4 °C overnight. Preparation of 5-μm serial sections, hybridization, washing, and detection were carried out as previously reported [32,33]. No positive signals were observed when sense probes were used, confirming the specificity of hybridization. The data were confirmed by three experiments using independent ovary samples.

2.4. PCR primers

All PCR primers are ordered from Sigma Aldrich Japan (Tokyo, Japan).

2.5. Real-time PCR of ovarian protease genes

Total RNA was isolated from the half-portion of the *Ciona* ovary incubated with vehicle (sterile seawater) alone or 1 μM Ci-TK as previously described [3]. Each ovary was incubated at 16 °C for 2, 5 or 15 h. The *Ciona* ovary RNA was reverse-transcribed to the template cDNA at 50 °C for 50 min using the oligo (dT) anchor primer and Superscript III first Strand Synthesis Supermix (Invitrogen). The real-time PCR for the gene expression of Ci-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Ci-cathepsin D, Ci-carboxypeptidase B1, and Ci-chymotrypsin was performed using Power SYBR Green PCR Master Mix and ABI Prism 7000 (Applied Biosystems, Foster City, CA) as previously reported [3]. In brief, Ct values for the Ci-GAPDH gene expression were used as standard values, and $\Delta\Delta$Ct values for each of the protease genes were calculated according to the manufacturer's instruction. The primers used for the real-time PCR are listed in Supplemental Table 1.

2.6. Measurement of protease activity in the ovary

The half-piece ovary was incubated in sterile seawater with Ci-TK at 18 °C for 1, 3, 6 and 16 h. The Ci-TK-treated or untreated ovaries were homogenized in 50 mM Tris HCl (pH 7.6) (for the total protease assay) or Cathepsin D assay buffer (Sigma, St. Louis, MO, USA, for the Cathepsin D assay), and centrifuged at 15000 × *g* at 4 °C for 5 min. The supernatants were frozen with liquid nitrogen, and stored at −80 °C until use. Measurement of protease activity of Ci-carboxypeptidase and Ci-chimotrypsin in the *Ciona* ovary extracts was performed as previously described [3,29]. The protein concentrations of the samples were determined using a BGA protein Assay Reagent Kit (Pierce, Rockfold, IL, USA) with bovine serum albumin as the standard. The Cathepsin D activity was quantified using the Cathepsin D Assay Kit (Sigma) according to the manufacturer's instruction. Each point represents the mean ± S.E.M. for seven samples. $^*P < 0.05$, compared with the enzyme activity at 0 h.

3. Results

3.1. Distribution of Ci-TK-upregulated proteases in the Ciona ovary

In the *Ciona* ovary, the large portion is occupied by preGVBD oocytes which are readily classified into three major growth stages on the basis of their diameter and organization of oocytes complexes: stage I (pre-vitellogenic stage), stage II (vitellogenic stage), and stage III (post-vitellogenic stage) [4,25]. Our previous study demonstrated that Ci-TK induced the activation of ovarian chymotrypsin, carboxypeptidase B1, and cathepsin D, which in turn

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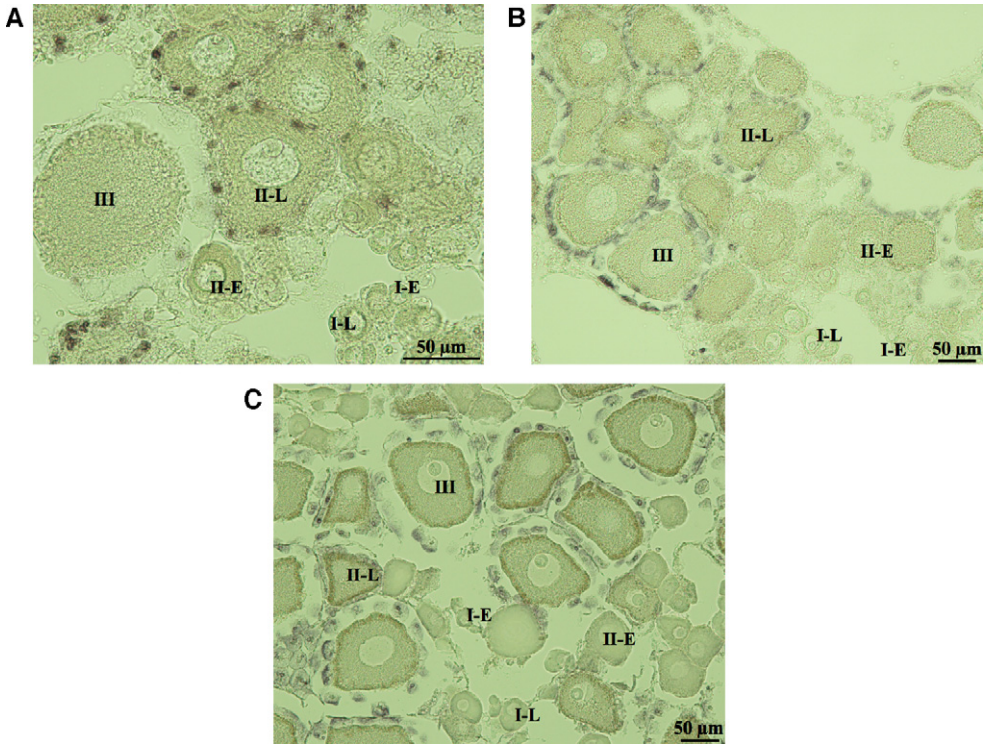

Fig. 1. Localization of the Ci-cathepsin D (A), Ci-carboxypeptidase B1 (B), and Ci-chymotrypsin (C) mRNAs in the *Ciona* ovary is shown. I-E, early-previtellogenic (early-stage I) oocytes; I-L, late-previtellogenic (late-stage I) oocytes; II-E, early-vitellogenic (early-stage II) oocytes; II-L, late-vitellogenic (late-stage II) oocytes; and III, postvitellogenic (stage III) oocytes. Scale bars in (A–C), 50 μm.

enhanced the growth of stage II oocytes [3]. To clarify the relationship between the localization of Ci-TK-R and that of the proteases, we initially performed in situ hybridization on the *Ciona* ovary. As shown in Fig. 1A, the Ci-cathepsin D mRNA was present exclusively in test cells of the stage II oocytes, which are believed to be functionally related to mammalian granulosa cells and to be involved in the growth of oocyte bodies and follicle cells [3,4,13], whereas Ci-carboxypeptidase B1 and Ci-chymotrypsin mRNAs were specifically detected in follicular cells of the stage II and stage III oocytes (Fig. 1B and C). On the other hand, no positive signal was detected using sense probes (Supplemental Fig. 1).

Ci-TK-R was also shown to be present exclusively in test cells of stage II oocytes [3]. To examine whether Ci-TK-R colocalized with Ci-cathepsin D, we performed double-immunostaining using Ci-TK-R and Ci-cathepsin D antibodies. The immunostaining of Ci-cathepsin D detected the localization of Ci-cathepsin D exclusively in the test cells of stage II oocytes (Fig. 2A). In contrast, no significant immunoreactivity was observed in any tissues of the ovary. These results are compatible with the in situ hybridization of the Ci-cathepsin D (Fig. 1A). Moreover, the double-immunostaining demonstrated that the immunoreactivity of Ci-TK-R was largely colocalized with that of Ci-cathepsin D (Fig. 2C), whereas no immunostaining was observed in test cells of oocytes at other stages (Fig. 2B). In addition, no positive signal was observed using antigen-absorbed antibodies (Fig. 2D), indicating the specificity of immunostaining. Collectively, these data confirmed the colocalization of Ci-TK-R and Ci-cathepsin D in the test cells of stage II oocytes.

3.2. Time-course changes in gene expression of ovarian protease in the presence of Ci-TK

To examine the gene expression profile of Ci-cathepsin D, Ci-carboxypeptidase B1, and Ci-chymotrypsin, real-time PCR was performed. The expression of a housekeeping gene, Ci-GAPDH gene, was not affected, as previously reported [3]. As shown in Fig. 3 and Supplemental Table 2, the alteration in the ovarian protease gene expression by Ci-TK was evaluated during the 15-h incubation period. The Ci-cathepsin D gene expression was detected at 2 h after treatment with Ci-TK, elevated for up to 5 h, and then slightly decreased at 15 h of incubation (Fig. 3A and Supplemental Table 2). As depicted in Fig. 3B and C, Ci-carboxypeptidase B1 and Ci-chymotrypsin gene expressions were detected at 5 h from induction of Ci-TK, and then decreased. These gene expression profiles suggest that the Ci-cathepsin D gene is initially upregulated, followed by the rapid upregulation of the other protease genes (Fig. 3 and Supplemental Table 2).

3.3. Time-course changes in ovarian protease activity in the presence of Ci-TK-I

We determined the activity profiles of the proteases upregulated by Ci-TK. The lysosomal protease Ci-cathepsin D is known to exert its maximal activity under a mildly acidic condition (pH 4.0), whereas neutral pH (pH 7.6) is optimal for Ci-chymotrypisn and Ci-carboxypeptidase B1 activity. Thus, we observed the acidic protease and neutral proteases in separate experiments. Fig. 4 shows the alteration in the ovarian protease activities induced by Ci-TK during the 16-h incubation period. Ci-cathepsin D activity was detected at 3 h after treatment with Ci-TK; it was elevated for up to 6 h, and then slightly decreased at 16 h of incubation (Fig. 4B). The 6-h Ci-TK-incubated ovary elicited an approximately 5.5-fold increase of Ci-cathepsin D activity compared to the ovary untreated with Ci-TK (Fig. 4A). As depicted in Fig. 4D and F, Ci-carboxypeptidase B1 and Ci-chymotrypsin activities elevated for up to 6 h from induction of Ci-TK, and then decreased. The 6-h Ci-TK-incubated ovary elicited an approximately 5.6 and 4.3-fold increase of the Ci-carboxypeptidase B1 and Ci-chymotrypsin activities,

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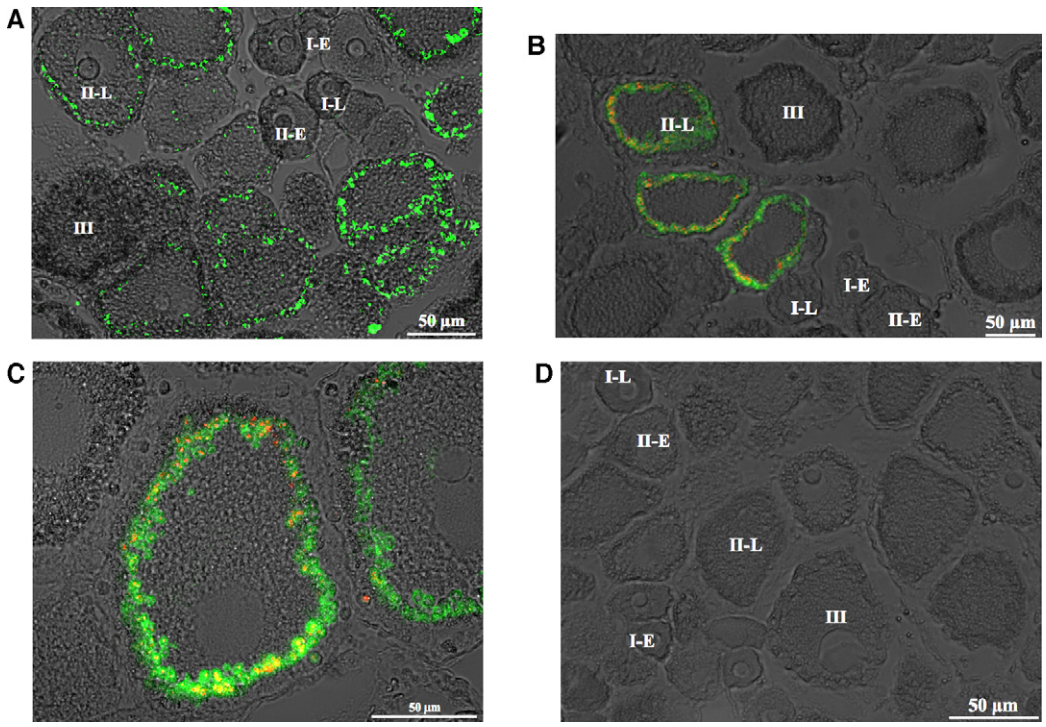

Fig. 2. Localization of Ci-cathepsin D and Ci-TK-R in the ovary. (A) Immunostaining of Ci-cathepsin D on a section of the ovary. (B) Double-immunostaining of Ci-cathepsin D (green signals) and Ci-TK-R (red signals) on a section of the ovary. (C) Immunostaining of Ci-cathepsin D (green signals) and Ci-TK-R (red signals) in test cells of the stage II-L oocytes. The merged image shows the co-localization of Ci-cathepsin D and Ci-TK-R in test cells of the stage II-L oocytes. (D) No positive signal was observed using the antigen-absorbed Ci-cathepsin D and Ci-TK-R antibodies. Scale bars in (A–D), 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

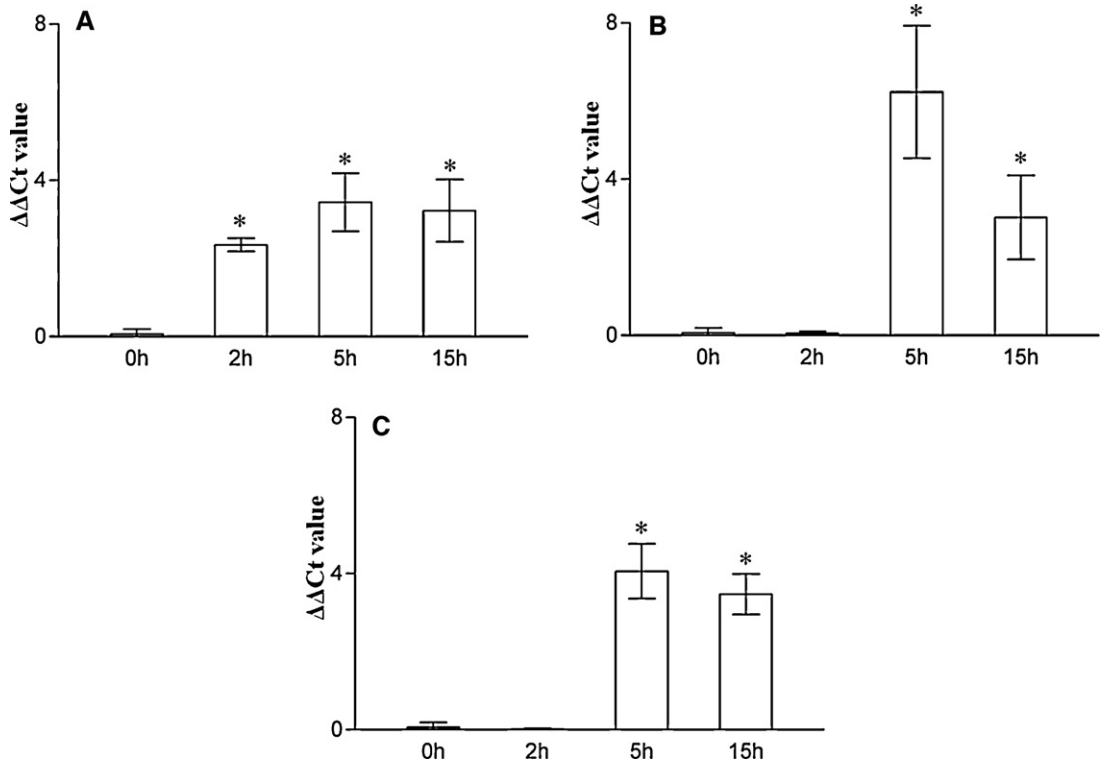

Fig. 3. Real-time PCR-based quantification of the gene expression of Ci-cathepsin D (A), Ci-carboxypeptidase B1 (B), and Ci-chymotrypsin (C) genes in the ovary treated with Ci-TK. The induction level of each gene expression regulated in the ovary treated with Ci-TK was calculated from the ΔΔCt values (see Supplemental Table 2). Each point represents the mean ± S.E.M. for three independent experiments. *$P < 0.05$, compared with the ΔΔCt value at 0 h.

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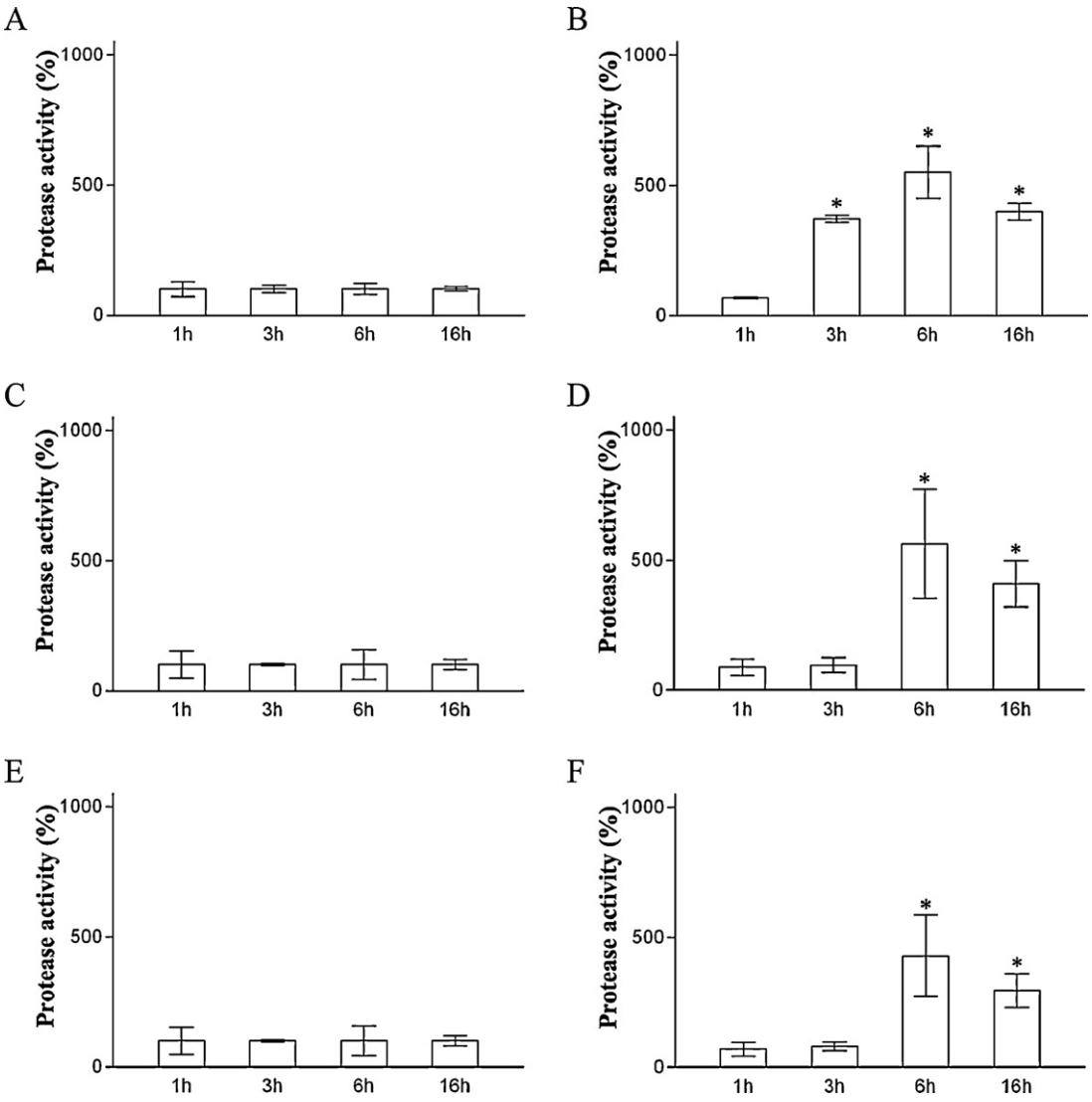

Fig. 4. Time course for the ovarian protease activities. The Ci-cathepsin D (B), Ci-carboxypeptidase B1 (D) and Ci-chymotrypsin (F) were up-regulated in the presence of 1×10^{-7} M Ci-TK-I, whereas there was no alteration in any protease in the absence of Ci-TK (A, C, and E). Each point represents the mean ± S.E.M. for seven samples. $^{*}P < 0.05$, compared with the enzyme activity at 0 h.

respectively, compared to the ovary untreated with Ci-TK (Fig. 4C and E). Notably, unlike the Ci-cathepsin D activity, the elevation of carboxypeptidase B1 and chymotrypsin activity was not detected at 3 h, but the initial and maximal activities were measured at 6 h after the treatment with Ci-TK (Fig. 4C–F).

4. Discussion

In the present study, the localization and activation profile of the three proteases responsible for the growth of *Ciona* oocytes from stage II to stage III were elucidated. Ci-cathepsin D was found to be localized to test cells (Fig. 1). In contrast, Ci-chymotrypsin and Ci-carboxypeptidase B1 are expressed in follicular cells (Fig. 1). Such distinct localization of Ci-cathepsin D, Ci-carboxypeptidase B1, and Ci-chymotrypsin mRNAs indicates that they are involved in the processing of different substrates. This view is also compatible with the findings that these proteases have different substrate selectivity and that cathepsin D is an acidic (aspartate) protease, while carboxypeptidase B1, and chymotrypsin are neutral metal and serine proteases, respectively [6,11,34,35].

Our previous study demonstrated that the prominent morphological changes of stage-II oocytes occurred at 6 h after treatment with Ci-TK [3]; the size of oocytes gradually increased, mitochondria were dispersed to the periphery of the cell membrane of oocytes, and the follicles gradually grew and formed a petal-like sharp shape. In the present study, Ci-cathepsin D, Ci-carboxypeptidase B1, and Ci-chymotrypsin were found to exhibit their maximal activities at 6 h after treatment with Ci-TK (Fig. 4). Consequently, the protease activity profiles (Fig. 4) are in good agreement with the growth of the oocytes from stage II to stage III.

Cathepsin D participates in the peoteolytic processing of vitellogenin into yolk proteins in vertebrates [6], indicating that Ci-cathepsin D is also involved in the processing of *Ciona* vitellogenin. Furthermore, this notion is consistent with the specific expression of Ci-cathepsin D in test cells of the vitellogenic oocytes (Fig. 1A). In vertebrates, vitellogenin is synthesized as a precursor of yolk protein in the liver, and is then transported to oocytes [10,12]. Quite recently, the C-terminal domain of vitellogenin was identified in the tissues corresponding to the liver in another ascidian species, *Halocynthia roretzi*, and the protein was found to be accumulated in the somatic body of oocytes [1]. In keeping with these findings, the localization of the Ci-cathepsin D mRNA and protein

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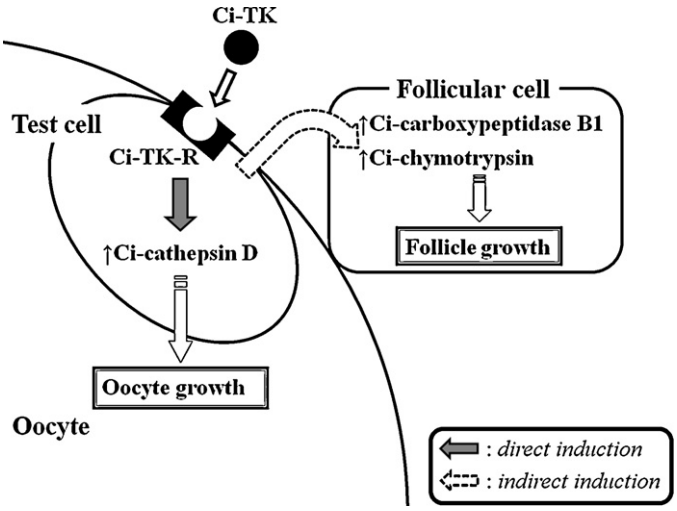

Fig. 5. Schematic diagrams of the protease activation mechanism underlying the tachykininergic protease-directed oocyte growth. Ci-TK activates Ci-TK-R specifically expressed in test cells of vitellogenic oocyte, and then directly up-regulates the transcription and enzymatic activity of Ci-Cathepsin D, followed by indirect (secondary) up-regulation of the transcription and enzymatic activities of Ci-carboxypeptidase B1 and Ci-chymotrypsin in follicular cells.

(Figs. 1 and 2) to the test cells, but not to the somatic body of the vitellogenic oocytes, led to the presumption that *Ciona* vitellogenin is transported into and processed by Ci-cathepsin D in the test cells followed by accumulation of mature yolk protein in the stage II oocytes, although no *Ciona* vitellogenin homologs have ever been characterized. Characterization of *Ciona* vitellogenin is expected to contribute to further understanding of Ci-TK-induced vitellogenesis by Ci-cathepsin D.

Ci-chymotrypsin and Ci-carboxypeptidase B1 were shown to be expressed exclusively in the follicular cells, which surrounded the stage II and III oocytes in the course of oocyte growth. In mice, carboxypeptidase B1 was involved in the production of major components of the zona pellucida surrounding oocytes [11]. These findings are compatible with the localization of Ci-carboxypeptidase B1 to follicular cells, and suggest that Ci-carboxypeptidase B1 is responsible for the proteolytic processing of the vitelline coat component proteins. No endogenous substrates of chymotrypsin during oocyte growth have ever been identified, although the involvement of chymotrypsin in the oocyte growth of various invertebrate species is suggested [9,30,34,35]. To further examine the molecular mechanisms underlying the protease-directed oocyte growth, the characterization of the authentic substrates of these proteases in *Ciona* is currently underway.

Also of interest is the regulatory mechanism underlying the activation of the proteases. The co-expression of Ci-cathepsin D with Ci-TK-R in test cells (Fig. 2) strongly suggests that Ci-cathepsin D is directly up-regulated by Ci-TK. In addition to the localization of Ci-cathepsin D, Ci-carboxypeptidase B1 and Ci-chymotrypsin, of particular significance is that induction of the Ci-cathepsin D gene was detected at 2 h-incubation with Ci-TK, and reached maximal induction at 5 h, whereas the activity of neither Ci-carboxypeptidase B1 nor Ci-chymotrypsin were detected at 2 h, but their initial and maximal induction was also observed at 5 h (Fig. 3). Furthermore, the protease activity showed very similar time-courses with 1-h time lag (Fig. 4). These results demonstrate that the activation of Ci-cathepsin D preceded those of Ci-carboxypeptidase B1 and Ci-chymotrypsin, but that Ci-carboxypeptidase B1 and Ci-chymotrypsin quickly activated. In combination, these profiles of gene expression and enzymatic activity of the three proteases lead to the following putative activation cascade (Fig. 5). (i) Ci-TK directly up-regulates induction of Ci-cathepsin D, (ii) subsequently, Ci-carboxypeptidase B1 and Ci-chymotrypsin undergo secondary and/or indirect activation in follicular cells. In addition, our previous study showed that only one inhibitor for each of the proteases completely blocked both the growth of the stage II oocyte bodies and of follicular cells, suggesting that the proteases are functionally concerted for the Ci-TK-induced oocyte growth [3]. Further investigation of the protease activation mechanism for the tachykininergic protease-directed oocyte growth, including characterization of putative secondary signaling molecules, will lead to a crucial clue to the understanding of the evolutionary conservation and diversity of biological roles of test cells and granulosa cells [4,13].

In conclusion, we have elucidated the localization and activity profile of three proteases, cathepsin D, carboxypeptidase B1 and chymotrypsin in the vitellogenic oocytes of *C. intestinalis*. Our present data lead to the exploration of the novel molecular mechanism and the evolutionary aspects of oocyte growth.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2011.07.019.

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