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Proteomic profiles of embryonic development in the ascidian *Ciona intestinalis*

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Abstract

We report here proteomics-based protein profiles of three embryonic stages of the ascidian *Ciona intestinalis*. Two-dimensional gel electrophoresis revealed 416, 539, and 695 protein spots in the unfertilized eggs, 16 cell-stage embryos, and tadpole larvae, respectively. Comparative and quantitative analyses of the spot patterns identified proteins showing an increase or decrease in amount during embryonic development. Protein identification by MALDI-TOF/MS indicated not only the abundance and importance of metabolic enzymes and translation elongation factors but also the functional importance of actin-binding proteins and molecular chaperones during ascidian development. Global changes in spots for vitellogenin-like protein suggested post-translational modification or proteolytic digestion of this protein during embryogenesis. Comparison between mRNA and protein levels among unfertilized eggs, 16 cell-stage embryos and tadpole larvae indicated nonparallel expression patterns of genes and proteins. Ascidians provide an excellent system for studying gene expression and cell differentiation during development, and the present study should shed light on the associated molecular mechanism at the protein level.

Key words

proteomics; *Ciona* embryo; two-dimensional gel electrophoresis; peptide mass finger printing; vitellogenin; larvae; egg; ascidian development

Introduction

Ascidians are primitive chordates and their evolutional position has received considerable attention for many years. Because of the simplicity and uniqueness of their developmental processes, they have provided excellent experimental systems for the study of developmental mechanisms (reviewed in Corbo et al., 2001; Satoh, 2001, 2003). The ascidian embryo undergoes a typical mosaic development (Conklin, 1905; Nishida, 1987). Autonomous specifications of the developmental fate of blastomeres are determined both by inheritance of maternal substances prelocalized to appropriate parts of the egg and by zygotic gene expression during development (Sardet et al., 2005). The ascidian tadpole larvae displays a primitive chordate body plan. For example, the simple nervous system, organized as a dorsal tubular nerve cord in tadpole larvae has served as a miniature system for studying the nerve system of vertebrates (Meinertzhagen and Okamura, 2001).

The ascidian *Ciona intestinalis* is a cosmopolitan species abundantly seen in a wide range of coastal habitats around the world. Analysis of the draft genome sequence of *C. intestinalis* has shown that it consists of ~ 160 Mbp with 15,852 protein-coding genes (Dehal et al., 2002). Extensive EST analysis was performed using cDNA libraries encompassing different developmental stages, including fertilized eggs, cleaving embryos, gastrulae/neurulae, tailbud embryos, tadpole larvae, and juveniles, and several tissues of mature adults, including the testis, ovary, heart, endostyle, neural complex and blood cells (Nishikata et al., 2001; Takamura et al., 2001; Fujiwara et al., 2002; Inaba et al., 2002; Ogasawara et al., 2002; Satou et al., 2002a, b). In addition, global analyses of gene expression profiles have been performed during embryogenesis by whole mount *in situ* hybridization (Satou et al., 2002a) and using large-scale cDNA or oligo-DNA microarrays (Azumi et al., 2003; Ishibashi et al., 2003; Yamada et al., 2005). These analyses of gene expression profiles at a transcriptional level provide insights into the spatio-temporal regulatory mechanism of ascidian gene expression during development (Imai et al., 2006).

Information on genome sequences serves as the molecular basis of proteomics. Protein profiles at certain stages of development and changes during development have been examined in multiple organisms, such as the mouse (Wilkinson et al., 1987; Murach et al., 1990; Latham et al., 1993; Greene et al., 2002), *Drosophila* (Gong et al., 2004) and zebra fish (Link et al., 2006). However, proteomic analyses in *Ciona* have been limited to a few studies on testis or sperm (Hozumi et al., 2004, 2006, 2008; Satouh et al., 2005; Inaba et al., 2007). Information from proteomic analyses is becoming indispensable in the study of development mechanisms in *Ciona* at the cell biology level. This information promises to improve the understanding of the functions of indivisual protein in *Ciona* development. In this study, we examined proteins expressed in unfertilized eggs, 16 cell-stage embryos, and tadpole larvae by two-dimensional gel electrophoresis (2-DE), and identified each protein by MALDI-TOF/MS. Comparative and quantitative analyses of the protein spot patterns revealed changes in specific proteins during development.

Materials and Methods

Chemicals and reagents

IPG strips and buffers were purchased from GE Healthcare (Buckinghamshire, UK). Actinase was obtained from Waken (Tokyo, Japan). α-Cyano-4-hydroxyciannamic acid (CHCA), and peptide calibration standards were purchased from Bruker Daltonics Japan (Tokyo, Japan). All the other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical (Osaka, Japan). Seawater used throughout this study was Millipore-filtered natural seawater.

Preparation of Ciona egg and larval samples

Ciona eggs and spermatozoa were directly collected from oviducts and sperm ducts, respectively, by dissection. To obtain unfertilized eggs, mature unfertilized eggs were dechorionated by a modification of a method described previously (Satou et al., 2001). In brief, seawater containing 10 mg/ ml sodium thioglycolate and 1 mg/ ml actinase was added to an egg suspension and incubated for 2-3 minutes at room temperature. The egg suspension was then pipetted and the eggs were collected by brief centrifugation with a hand-driven centrifuge. The eggs were washed five times with excess seawater and then spread on gelatin-coated plastic plates. To prepare 16 cell-stage embryos, the eggs were inseminated before spreading on the plates. One hundred dechorionated eggs or 16 cell-stage embryos were transferred into a wash solution composed of 1 M mannitol and 10 mM HEPES-NaOH (pH 8.0). After collection of eggs or embryos by brief centrifugation with a hand-driven centrifuge, 200 µl isolelectric focusing (IEF) lysis buffer (8 M urea, 2 M thiourea, 0.1% Triton X-100, 10% 2-propanol, 4% CHAPS, 50 mM DTT) was added to the pellet, following which the proteins were allowed to solubilize for 40 minutes at room temperature while being mixed using a rotator.

To prepare tadpole larvae, eggs were inseminated and incubated for 18 h at 18ºC and were allowed to hatch naturally. One hundred hatched tadpole larvae were transferred to a single tube and collected by centrifugation at 5,000 g for 10 min at 4ºC. Proteins in the packed larvae were solubilized in 200 µl IEF lysis buffer.

2-DE

Two-hundred microliters of lysate from 100 specimens at each developmental embryonic stage was supplemented with an appropriate IPG buffer (final concentration 0.5%) (GE Healthcare) and applied to pH4-7 or pH 6-11 IPG strips 11 cm in width. IEF was performed with the following voltage programs on an Ettan IPGphor III IEF unit (GE Healthcare): for pH4-7 gel strip, 10 h re-hydration and subsequent focusing at 30 V for 10 h step-and-hold, 200 V for 1 h gradient, 500 V for 1 h gradient, 1000 V for 1 h gradient, 8000 V for 30 min gradient, and 8000 V for 6h step-and-hold, and for pH6-11 gel strips, 30 V for 14 h step-and-hold, 200 V for 1 h step-and-hold, 500 V for 1h gradient, 1000 V for 1h step-and-hold, 2000 V for 1h step-and-hold, 3000 V for 1 h step-and-hold, 8000 V for 1 h gradient, and 8000 V for 10 h step-and-hold.

After completion of IEF, the gel strips were equilibrated with SDS-PAGE sample buffer containing 6 M urea and 50 mM DTT for 40 min; this was followed by second dimension SDS-PAGE on a 10% polyacrylamide gel (Laemmli, 1970). Protein spots were visualized by silver staining (Silver Stain Kit II; Wako Pure Chemical Industries). For peptide mass fingerprinting by MALDI-TOF/MS, the gel was stained with Coomassie Brilliant Blue R-250.

Image analysis of 2-DE gel

Silver-stained 2-DE gels were scanned and digitized with a GS-710 calibrated densitometer scanner (Bio-Rad, Hercules, CA, USA). Protein gel analysis was performed using the commercial software, PDQuest ver. 5 (Bio-Rad). For each gel, the digitized image was smoothed and spot peaks were detected. Spot peaks on each gel

were aligned and matched along the positions of these peaks, and the protein spots were numbered as Spot Specific Number SSN. To analyze the variation in protein spots among different developmental stages, protein quantities (QTY) were calculated as averages of normalized peak values of protein spots in triplicate gels. The QTY value was divided by the relative molecular weight on SDS-PAGE to obtain the relative molar ratio (QTY/MW).

In-gel digestion of proteins

Since the amount of protein in silver-stained gels was not sufficient for peptide mass finger printing analysis by MALDI-TOF MS, 2-DE was performed with an approximately 10-fold increased amount of protein. The gels were stained with Coomassie Brilliant Blue R-250. Protein spots were excised using large-diameter polystylene-tips with a micropipettor, followed by tryptic digestion as follows: small pieces of protein spot gel (approximately 1.5 mm cubes) were sequentially soaked in 50% methanol twice for 10 min each and in 100% acetonitrile for 5 min. Dehydrated gel pieces were dried and the gels were then re-hydrated with trypsin solution (10 ng / µl trypsin in 100 mM NH_4HCO_3) for 15 min. Excess trypsin solution was removed and the gel pieces were covered with 100 mM NH_4HCO_3 and incubated for 12 h at 37ºC. Eluted peptides were directly subjected to MALDI-TOF/MS or MALDI-TOF/TOF MS analysis.

MALDI-TOF/MS and MALDI-TOF/TOF MS analysis

A layer of matrix (CHCA) was prepared in advance on a MALDI target plate (Anchor Chip™; Bruker Daltonics, Billerica, MA, USA). For each sample, 1 µl of peptide solution was mixed with 1% TFA and spotted over the matrix on the target plate. After 3 min of incubation, samples were washed with 0.1% TFA. An Autoflex II system (Bruker Daltonics) was operated in the reflector-positive ion mode for most peptide samples for PMF analysis. Some samples were analyzed by MALDI-TOF/TOF with MS/MS analysis under post-source decay or

collision-induced decay.

The PMF or MS/MS spectra obtained were manually submitted to the MASCOT server (http://www.matrixscience.com/; Matrix Science, London, UK) using MS spectrum analysis software (Biotools; Bruker Daltonics). Mass searching by MASCOT was performed with mass tolerance of ±100 or ±150 ppm for PMF and ±0.2 Da for MS/MS or MS. Oxidation of methionine was considered as a variable modification. For protein identification, protein databases of *C. intestinalis* from the JGI ver. 2.0 gene model (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html) and from KYOTOGRAIL2005 (http://ghost.zool.kyoto-u.ac.jp/indexrl.html) were used. The proteins deduced from the latter were integrated into the protein database called CIPRO, and each protein was assigned a CIPRO ID. The PROCITS database (Hozumi et al., 2004) was also used.

Data analysis and protein identification

Gene models identified by PMF or MS/MS analysis were further used in BLASTP search of the NCBI non-redundant protein database. Classification of proteins identified by PMF or MS/MS analysis was performed according to Tatusov et al. (2003) with modifications. Some of the identified proteins were compared with expression profiles from DNA microarrays developed recently (Azumi et al., 2007) and with EST expression profiles in the *C. intestinalis* cDNA databases (http://ghost.zool.kyoto-u.ac.jp/indexrl.html). Some data for EST counts were kindly provided by Dr. Yutaka Satou (Kyoto University, Kyoto, Japan).

Results and Discussions

Separation of egg and embryonic proteins by 2-DE

To obtain an overview of protein expression patterns and their global changes during embryogenesis in *C. intestinalis*, 2-DE was performed with unfertilized eggs, 16 cell-stage embryos and tadpole larvae. We used two different ranges of IPG strips, acidic (pH 4-7) and basic (pH 6-11) for first dimension IEF, and a 10% polyacrylamide gel for second dimension SDS-PAGE, which facilitated separation of proteins over a wide range of pI and was suitable for detecting global changes in proteins during embryogenesis. Triplicate gel electrophoresis in each pH range was performed in unfertilized eggs, 16 cell-stage embryos and tadpole larvae. Silver-stained gels showed reproducible patterns of protein spots. Representative gel images of the three developmental stages are shown in Fig. 1. These gel images were statistically analyzed using PDQuest software, resulting in the validation of 416, 539, and 695 spots in unfertilized eggs, 16 cell-stage embryos, and tadpole larvae, respectively. These protein numbers represent major components rather than total protein numbers in each developmental stage. Therefore, comparison of these gels could lead to knowledge about major changes in protein composition during development. Image analysis and subsequent statistical analysis using PDQuest demonstrated the identity of protein spots among the three different developmental stages. The quantity of each protein spot was estimated by normalization of spot intensity in each gel using PDQuest. Average quantities were calculated from three gel sets as normalized quantities (Supplementary Table S1).

Protein identification

We subjected 199, 153, and 624 major protein spots from unfertilized eggs, 16 cell-stage embryos, and tadpole larvae, respectively, to protein identification. Peptide mass fingerprinting with MALDI-TOF MS identified 154, 96, and 324 proteins in unfertilized eggs, 16 cell-stage embryos, and tadpole larvae, respectively. These

proteins are listed with detailed information including matching scores and BLAST search results (Supplementary Table S2). Proteins identified in the three developmental stages were mostly involved in cellular housekeeping activities, such as metabolism, protein synthesis, protein degradation, intracellular traffic, protein folding, cytoskeletal organization and cell proliferation. These proteins were classified by their cellular function, according to the KOG classification (Tatusov et al., 2003), with some modifications (Fig. 2 and Supplementary Table S2).

The relative molar ratio (QTY/MW) of each protein was calculated from its quantity divided by the molecular weight observed on SDS gels. Values of QTY and MW were estimated from the position and intensity of each spot obtained by PDQuest. Comparison of summed QTY/MW values in each KOG cluster during embryonic development revealed overall changes in protein populations. The most abundant protein in unfertilized eggs was a yolk protein, vitellogenin (described below), which clustered independently in this study (Fig. 2). The quantity of vitellogenin decreased during development to the tadpole larval stage. It is intriguing that cluster S (function unknown) decreased during cleavage, suggesting that several unknown proteins exist in unfertilized eggs as maternal proteins.

Cluster O, including chaperones and heat shock proteins, decreased from the unfertilized egg stage to 16-cell embryo stage, but increased again from the 16 cell-stage embryo to the tadpole larval stage. The abundance of heat shock proteins in developing embryos has been described in sea urchins, mice and zebra fish from proteomic analyses (Greene et al., 2002; Link et al., 2006; Roux et al., 2008). The molecular chaperones, HSP70, HSP60, and HSP90, and the subcomponents of chaperones containing t-complex protein (TCP-1) are ubiquitous molecules among eukaryotes and are found throughout embryonic development in *C. intestinalis*. In particular, TCP-1 protein is known to direct folding of cytoskeletal proteins, such as α, β, and γ-tubulin, actin and centractin (Liang and MacRae, 1997). Subunits of TCP-1 showed significant decreases from the unfertilized egg stage to 16-cell embryo stage but showed no further change in tadpole larvae (Supplementary Table S2). *C.*

intestinalis has several genes for the HSP chaperone system (Wada et al., 2006). HSP70 may have a function in gamete recognition as a component of a self-sterility mechanism (Marino et al., 1998, 1999). Stress-induced phosphoprotein 1 (STIP1) homolog was also expressed in *Ciona* tadpole larvae. STIP1 also functions as a molecular chaperone and has been associated with HSP70 and HSP90 (Scheufler et al., 2000). At the protein level, both HSP70 and STIP were expressed in unfertilized eggs, but showed a decrease during development to the 16-cell embryo stage and again increase to the tadpole larval stage (Supplementary Table S2). This could be related to the fact that HSP90/NOS enhances tail resorption at the onset of metamorphosis and that inhibition of HSP90 arrests morphogenesis of tadpole larvae in the ascidians *Boltenia villosa* and *Cnemidocarpa finmarkiensis* (Bishop et al., 2002). Another molecular chaperone, a protein disulfide isomerase, is thought to play a role in glycoprotein biosynthesis in combination with other co-factors in the ER lumen (Oliver et al., 1997). Several protein disulfide isomerases and their associated proteins were also found in *C. intestinalis* but most of them increased from the unfertilized egg stage to the tadpole larval stage (Supplementary Table S2; ; see also Table 6).

Several subunits of 26S proteasomes were categorized into cluster O. In ascidians, proteasomes are required for sperm/egg-binding (Sawada et al., 2002) and cell cycle regulation during blastomere cleavage (Kawahara and Yokosawa, 1994). During subsequent metamorphosis, apoptosis-mediated degradation is reported to be involved in absorption of the tail (Chambon et al., 2007). Many of the proteasomal subunits showed an increase from 16-cell embryo stage to tadpole larval stage (Supplementary Table S2), suggesting that they are involved in metamorphosis.

The clusters C, G and H included proteins involved in energy metabolism. Most of the proteins in these clusters showed increases during embryonic development but the size of the increases was relatively small. Seventy-one protein spots in these clusters represented mitochondrial proteins, mostly concerned with ATP synthesis; 10 were components of ATP synthase; 14 were concerned with the β-oxidation cascade, 18

were concerned with the electron transfer cascade of oxidative phosphorylation, and 29 were TCA cycle enzymes. The abundance of proteins in the β-oxidation cascade suggests the importance of ATP production utilizing fatty acids, possibly by the yolk protein vitellogenin. Coenzyme A-related proteins and the components of ATP synthase are known to be up-regulated in the developing embryo of the mouse (Greene et al., 2002). Ascidian mitochondrial proteins, however, showed only slight changes in quantity during development, compared with those in mouse.

Cluster Z included cytoskeletal proteins and increased during development to the tadpole larval stage. During the early stages of ascidian development, dynamic changes in the organization of the cytoplasm occur in the cytoskeletal elements (Sardet et al., 2005). Ascidian embryos develop into tadpole larvae, the muscles of which constitute the motile compartment of the tail. Both in eggs and tadpole larvae, tubulins, microtubule-associate proteins, actin and many actin-binding proteins were found, suggesting the participation of these proteins in above cytoskeleton-related events (also discussed later).

Signaling molecules categorized in cluster T (Fig. 2) were mainly small G-protein-related proteins. Among them, Ran GTPase was highly expressed during embryonic development (Supplementary Table S2). It is notable that a protein showing similarity to human GTP-binding protein PTD004 isoform 1 was less expressed in both the unfertilized egg and 16 cell-stage embryo but highly expressed in the tadpole larva (Supplementary Table S2), suggesting that it plays a role in differentiated larval tissues.

The gene transcriptional network during early embryogenesis has been widely studied in *C. intestinalis* (Imai et al., 2006). In the present study, two proteins in cluster K (transcription) were detected at the protein level. One was SSN#A6605 (assigned as CLSTR05520 in the *Ciona* cDNA database at http://ghost.zool.kyoto-u.ac.jp/indexr1.html) and the other was SSN#A8607 (CLSTR00255, Ci-FUSE) (Imai et al., 2004). The former is a transcription factor and showed an almost constant quantity during development (Supplementary Table S2).

The latter is an RNA-binding protein with three KH-I RNA-binding sites and showed a decrease from the unfertilized egg stage to 16-cell embryo stage (Supplementary Table S2), although it was not significant due to the coefficient of variance (C.V.). Intriguingly, the level of Ci-FUSE mRNA was consistently high from the unfertilized egg to the tailbud embryo stage (33/31209 EST counts) but markedly low in the larva (2/24532 EST counts), suggesting translational regulation of this gene in the differentiation of larval tissues.

Cluster J (translation and ribosomes) increased from the unfertilized egg stage to the 16-cell embryos, but decreased from the 16-cell embryo stage to the tadpole larval stage. In particular, the highly abundant protein translation elongation factor 1A (eF1A) increased from the unfertilized egg stage to the 16-cell embryonic stage (Supplementary Table S2; see also Table 6). Between 6 and 10 min after fertilization, translation increases 5-fold in sea urchin eggs (Epel, 1967). Recently the level of a subunit of eF1B was shown to increase by 2 min after fertilization in a proteomic study on in sea urchin eggs (Roux et al., 2008).

Yolk protein

Multiple protein spots were found to show similarities to apolipoprotein B (Supplementary Table S2). Apolipoprotein B is a member of the large lipid transfer protein family. This protein family includes vitellogenin, a major component of egg yolk, which is a mixture of materials used for embryonic nutrition. Based on the alignment of protein IDs using a JGI gene model (Fig. 3), we consider that the apolipoprotein B is a *Ciona* vitellogenin (Ci-Vtg).

The gene model JGI_286147, encoding vitellogenin (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html), was found on chromosome 8 in the *C. intestinalis* genome. This gene model overlapped with three protein models in CIPRO; CIPRO.100.11.1, CIPRO.100.2.1 and CIPRO.100.7.1 (Fig. 3). Peptide mass fingerprinting showed that many spots on the 2-DE gel coincided with these three gene models. Based on the the gene model JGI_286147, Ci-Vtg is considered a

protein with molecular masses of 478 kDa and pI 8.0. However, the largest vitellogenin spots in unfertilized eggs were detected at around ~200 kDa (Fig. 4A, rectangle), suggesting the processing of this protein. Moreover vitellogenin proteins were detected in multiple spots on 2-DE gel in unfertilized egg, 16 cell-stage embryos and tadpole larvae (Fig. 4). These spots were widely dispersed on the 2-DE gel, but appeared to be mainly distributed around ~200 kDa, ~110 kDa, ~60 kDa, ~40 kDa and ~30 kDa in unfertilized eggs (Fig. 4A). During development, especially from the 16 cell-stage embryos to the tadpole larvae, Ci-Vtg became appeared in an increasing number of spots. In tadpole larvae, the major ~200 kDa cluster had diminished markedly and other vitellogenin spots became more scattered in the 2-DE gel (Fig. 4B, C). These results suggest that Ci-Vtg undergoes proteolysis during development. This is the first demonstration of evidence for vitellogenin processing in ascidians.

Vitellogenin is a protein synthesized in the liver and transported into vitellogenic oocytes by the bloodstream in vertebrates (Flickinger and Rounds, 1956; Danilchik and Gerhart, 1987). To establish where vitellogenin is expressed in *C. intestinalis*, EST counts were examined from several adult tissues (http://ghost.zool.kyoto-u.ac.jp/indexr1.html). ESTs were available only for KYOTOGRAIL2005.100.7.1 (CIPRO.100.7.1) from digestive glands or testes, with EST counts of 1/16219 and 1/4567, respectively. This result suggests that Ci-Vtg is synthesized in digestive gland and/or testis and is transported into oocytes in *C. intestinalis*.

Comparison between mRNA and protein levels

Ciona genes have been classified into five categories according to the stage at which a high level of expression occurs during the life cycle by the use of microarray analysis (Azumi et al., 2007). Among the proteins identified in this study, 221 (54 in unfertilized eggs, 41 in 16 cell-stage embryos, and 126 in tadpole larvae) could be assigned to the KYOTOGRAL2005 gene model. These proteins were classified into the categories in the microarray assay (Table 1). In unfertilized eggs, 59% of proteins

were classified into the maternal gene cluster, suggesting that maternal genes were expressed not only at the mRNA level but also at the protein level. Although the population of maternal gene products decreased from unfertilized egg stage to the tadpole larval stage, they were still expressed and constituted a large part of the protein pool in 16 cell-stage embryo (59%) and tadpole larvae (42%). They may have included proteins that were expressed from maternal mRNA during development. On the other hand, the embryonic gene cluster shows a pattern of high zygotic gene expression levels during embryogenesis (Azumi et al., 2007). The population of these gene products in unfertilized egg or 16 cell-stage embryo, however, was quite small in our study (6 or 0%, respectively). The population of the products from embryonic/adult gene cluster was small in the unfertilized eggs (9%), but that from 16 cell stage-embryos increased to 15 % (Table 1). In tadpole larvae, the proteins of these clusters were present at higher level; 10 or 20% of the population showed the embryonic gene and embryonic/adult gene clusters, respectively (Table 1). This suggests that most proteins in this category are gene products from mRNA of zygotic genes. Intriguingly, the protein population of the adult gene cluster decreased from unfertilized egg stage to tadpole larval stage (20% to 15%). This occurs because the adult gene cluster contains genes that show high expression in both embryos and adults. Therefore the protein products in this category may be mostly composed of maternal proteins.

Proteins abundantly present in unfertilized egg, 16 cell-stage embryo and tadpole larvae are listed in the order of QTY/MW (Table 2). Multiple spots of vitellogenin are listed; the sum of their QTY/MW reached ~3500, indicating that vitellogenin was the most abundant protein in unfertilized egg. The protein spot with highest QTY/MW was SSN#A7109 in unfertilized egg. SSN#A6111 also showed high QTY/MW. These protein spots were subjected to peptide mass fingerprinting but they could not be identified. Many other abundant proteins in unfertilized eggs are involved in metabolism. In particular, subunits of ATP synthase accounted for a large part of protein pool in eggs and their QTY/MW values summed to ~870. Other

abundant proteins in eggs included actin, metabolic enzymes involved in glycolysis and molecular chaperones.

In 16 cell-stage embryos, a spot of translation elongation factor 1A showed the highest QTY/MW. This spot was highly abundant in unfertilized eggs or tadpole larvae (Table 2). The spot (SSN#A7109) showing highest QTY/MW value in unfertilized eggs mostly disappeared. The QTY/MW value of the spot (SSN#A6111) increased. Intriguingly, these two spots were completely lost in tadpole larvae (Table S1). Voltage-dependent anion-selective channel protein 2 (SSN#B7101) was abundant in both unfertilized eggs and 16 cell-stage embryos. Another spot of this protein (SSN#B7103) became abundant at 16-cell embryo stage (Table 2). This spot became predominant compared to the spot SSN#B7101 in tadpole larvae, suggesting an isoform replacement of this protein or post-translational modification.

Tadpole larvae still contained vitellogenins as the abundant components but their population was much smaller than that of eggs. From the unfertilized egg stage to the tadpole larval stage, high quantity of actin (spot SSN#A2209) was detected by 2DE (Table 2). This protein is one of the cytoplasmic types of actins (Chiba et al., 2003). In tadpole larvae, a cytoskeletal actin form (spot SSN#A1308; PROCITS10733.1.1.0), which was likely the main component of the tail muscle in tadpole larvae, was detected.

Large-scale EST analysis demonstrated the profiles of genes that are expressed in each developmental stage (Nishikata et al., 2001; Kusakabe et al., 2002). We examined the occurrence (number of clones per total cDNAs) of highly expressed genes and examined their correspondence to the gene products (Table 3). Most abundant gene among 29,444 cDNA clones from unfertilized eggs was the gene for cytoplasmic actin gene (CLSTR00046; Table 3). The product of this gene was present in a relatively high amount (Table 2). This was also the case in 16 cell-stage embryos and tadpole larvae. Another actin gene CLSTR00025 is also highly expressed in tadpole larvae. However, its gene product could not be detected by 2DE (Table 2; Table S2). The EST counts of this gene indicated its high expression in adult tissues

(http://ghost.zool.kyoto-u.ac.jp/indexr1.html). Therefore its mRNA might be synthesized and stored until the adult tissues differentiate. The product of CLSTR36868, on the other hand, was present in tadpole larvae in a relatively large amount (Table 2). This gene showed no expression in any adult tissues, suggesting that the gene product was specific to larval muscle.

The mRNA for Ci-Vtg was detected neither in unfertilized eggs, 16 cell-stage embryos nor tadpole larvae, because it was expressed in other tissues as described above. The products of genes with no sequence similarity to any known gene, such as CLSTR00424, 00032, 00065, 00087 and 00419, could not be detected as the protein spots in unfertilized eggs, 16 cell-stage embryos or tadpole larvae. This may be because these mRNAs represent maternal stocks destined for translation during the later stages of development. Thus, it may be concluded that the levels of mRNAs and proteins do not always correlate during development.

Proteins showing changes in quantity from unfertilized egg to tadpole larvae

To elucidate proteins specifically expressed at each embryonic stage, a master gel image was processed from triplicate 2-DE gels of unfertilized eggs, 16 cell-stage embryos and tadpole larvae, and this was followed by a comparison of protein spots using PDQuest. Protein spots that could be detected in only one developmental stage were regarded as unique to that stage. As a result, 5, 26, and 190 spots were verified as being unique in unfertilized eggs, 16 cell-stage embryos, and tadpole larvae, respectively. These proteins showing the high quantities are listed in Table 4 and their positions on 2DE gels are shown in Fig. 1.

Comparison of the quantity of each protein spot with that of the corresponding spot in another embryonic stage identified proteins showing marked changes in quantity during development. When the range of quantity changes was set to ± 20%, it was concluded that 145 and 288 spots increased and decreased the quantity from unfertilized egg stage to 16-cell embryo stage, respectively. On the other hand, 302 and 323 spots showed an increase and a decrease in the quantity, respectively, from

the 16-cell embryo stage to the tadpole larval stage. Proteins showing an increase in quantity during development were regarded as up-regulated proteins and those showing a decrease were regarded as down-regulated or maternal proteins.

Ci-Vtg was separated into multiple spots, possibly by posttranslational modification and degradation, during embryonic development. As a result, one Vg spot from the 16-cell embryo stage or three Vg spots from the tadpole larval stage were found in large quantity and were unique to each developmental stage (Table 4). Other Vg spots also included those showing up-regulated and down-regulated during embryonic development, especially from the 16-cell embryo stage to the tadpole larval stage (Table 5 and 6). As described above, quantities of the TCP-1 subunits decreased from the unfertilized egg stage to the 16-cell embryo stage (Table 6). Many spots showing marked changes in the quantities could not be identified by peptide mass fingerprinting (Table 4, 5 and 6), but the reason is not be elucidated. It is possible that no sequence data of these proteins were registered in the protein database for peptide mass fingerprinting. Alternatively, these proteins may be highly modified post-translationally.

The most remarkable feature in protein changes during development was observed in the regulatory proteins for actin dynamics or actin-based cell motility. A myosin light chain kinase (SSN#B3208) was uniquely identified in 16 cell-stage embryos. Non-muscle myosin II functions in the formation of contractile ring during cytokinesis. It is involved in the cleavage of early stage embryos in many organisms including *Drosophila* and sea urchins (Urven et al., 2006; Uehara et al., 2008). In ascidian embryos, a cytoskeleton-accumulated cortical structure called centrosome-attracting body (CAB) is involved in unequal cleavage of the embryos (Hibino et al., 1998). It has been reported that the localization of aPKC and ER network to the CAB is dependent on actin filaments (Patalano et al., 2006). The increase in the amount of actin-binding proteins, such as ezrin/radixin/moesin-like protein and fascin 1 (Table 5), may be related to the actin-mediated event during early stages of embryogenesis in ascidians.

Conclusion

Here we presented 2-DE protein patterns of unfertilized eggs, 16 cell-stage embryos and tadpole larvae in *Ciona intestinalis*. MS-based analyses of the spots identified major proteins in each developmental stage. Comparative and quantitative analyses allowed us to create a profile of the protein expression in each developmental stage and to obtain an overview of protein dynamics in the embryogenesis of *C. intestinalis*. We presume that the vast majority of proteins common throughout the development are housekeeping proteins, and the differences between these stages are seen in the proteins translated from both maternal and zygotic genes. Combining these proteomic analyses with the information from EST and microarray analyses led us to the idea that the levels of mRNAs and proteins do not always correlate. For some proteins, we found a large difference in the levels of mRNA and the corresponding protein product. This implies the existence of a sophisticated regulation system in the process of protein translation during development. Further functional analysis of each protein will unveil the regulatory mechanisms of embryogenesis, morphogenesis and metamorphosis.

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Figure legends

Fig. 1. 2DE protein profiles of the unfertilized egg stage, 16-cell embryo stage and tadpole larval stage. Each panel shows a representative 2-DE gel image from three experiments. (A) unfertilized eggs. (B) 16 cell-stage embryos. (C) tadpole larvae. Each set contains 2DE images with 4-7 IEF (left) and pH 6-11 IEF (right), visualized by silver. The spot positions of proteins listed in Tables 4, 5 and 6 are indicated: arrowheads, protein spots unique to the stage of embryogenesis indicated; closed arrows, protein spots showing a decrease in amount at the subsequent embryonic stage; open arrows, protein spots showing increase compared with previous embryonic stage. Numbers on each spot indicate SSN assigned by the software PDQuest and cited in supplementary in Table S1 and S2. The positions of molecular markers (kDa) are indicated on the left. Blanketed bars and numbers above the gels indicate pH range of IEF.

Fig. 2. Functional grouping of proteins identified in *Ciona* embryos, based on eukaryotic clusters of orthologous groups (KOG). Vitellogenin (Vg) is grouped independently. The pie charts were made based on the sum of QTY/MW values of the protein spots in each classification category. A, unfertilized eggs; B, 16-cell embryos; C, tadpole larvae. Letters in charts indicate categories of proteins identified in each developmental stage: C, energy production and conversion; D, cell cycle and mitosis; E, amino acid transport and metabolism; F, nucleotide metabolism and transport; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure and biogenesis; K, transcription; M, cell wall/membrane/envelope biogenesis; O, posttranslational modification, protein turnover and chaperone functions; P, inorganic ion transport and metabolism; S, function unknown; T, signal transduction mechanisms; Vg, vitellogenins; Z, cytoskeleton.

Fig. 3. *Ciona* vitellogenin. The JGI_286147 encodes a protein with 4,262 amino acids with a signal peptide and a vitellogenin domain. Protein sequences in CIPRO database are made, based on KYOTOGRAIL2005 in the Ghost database (http://ghost.zool.kyoto-u.ac.jp/indexrl.html). Six protein IDs in CIPRO database are mapped on JGI_286147. The IDs that could be identified by peptide mass fingerprinting in this study are shown in red.

Fig. 4. Distribution of vitellogenin spots in 2-DE gels from unfertilized eggs, 16 cell-stage embryos and tadpole larvae. Spots representing vitellogenin derivatives are indicated by open arrowheads. The vitellogenin spots showing the highest molecular mass are surrounded by a rectangle. (A) unfertilized egg; (B) 16 cell-stage embryo; (C) tadpole larvae. Each panel shows a 2-DE gel with IEF at pH 4-7 (left) and pH 6-11 (right). All gels were visualized using silver staining. Positions of molecular markers are indicated on the left (kDa).