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Further EST analysis of endocrine genes that are preferentially expressed in the neural complex of *Ciona intestinalis*: Receptor and enzyme genes associated with endocrine system in the neural complex

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Abstract

Identification of orthologs of vertebrate neuropeptides and hypothalamic hormones in the neural complex of ascidians suggests integral roles of the ascidian neural complex in the endocrine system. In the present study, we investigated endocrine-related genes expressed in the neural complex of *Ciona intestinalis*. Comprehensive analyses of 3′-end sequences of the neural complex cDNAs placed 10,029 clones into 4051 independent clusters or genes, 1524 of them being expressed preferentially in this organ. Comparison of the 1524 genes with the human proteome databank demonstrated that 476 matched previously identified human proteins with distinct functions. Further analyses of sequence similarity of the 476 genes demonstrated that 21 genes are candidates for those involved in the endocrine system. Although we cannot detect hormone or peptide candidates, we found 21 genes such as receptors for peptide ligands, receptor-modulating proteins, and processing enzymes. We then characterized the *Ciona* prohormone convertase 2 (Ci-PC2) and carboxypeptidase E (Ci-CPE), which are associated with endoproteolytic processing of peptide hormone precursors. Furthermore, genes encoding these transcripts are expressed specifically in the neural complex of young adult ascidians. These data provide the molecular basis for further functional studies of the endocrine role of the neural complex of ascidians.

Keywords: Ascidians; Neural complex; EST analysis; Endocrine system

1. Introduction

Ascidians (urochordates) occupy the most basal evolutionary position in the phylum of Chordata. *Ciona intestinalis* is one of the cosmopolitan ascidians. The draft genome of *C. intestinalis* is composed of 153–159 Mbp, containing 15,852 protein-coding genes (Dehal et al., 2002). This genome size and gene number is smaller than that of vertebrates, and comparable to that of *Drosophila melanogaster*. It is proposed that two gene-duplication events occurred early in the process of vertebrate evolution (Holland et al., 1994), while *C. intestinalis* is thought to possess chordate prototype genes. Thus, *Ciona* is an excellent simple model for evolutionary and functional studies of the endocrine system in chordates (Satoh et al., 2003; Campbell et al., 2004).

The neural complex of adult ascidians consists of a small cerebral ganglion and neural gland. The cerebral ganglion is a white spindle-shaped body that is forked at the anterior and posterior ends, from which paired nerves originate. The neural gland is an ovoid body of spongy texture lying immediately ventral to the ganglion (Chiba et al., 2004). Several reports have shown that the ascidian neural complex contains orthologs of vertebrate neuropeptides, hypothalamic

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hormones, and their receptors such as cholecystokinin and gastrin (Johnsen and Rehfeld, 1990; Nilsson et al., 2003), GnRH (Adams et al., 2003; Kusakabe et al., 2003; Tello et al., 2005), and tachykinin (Ci-TK) (Satake et al., 2004). Sherwood also reviewed existence of these ligands and receptors in the neural complex (Sherwood et al., 2005, 2006). These reports suggest that the neural complex plays an integral role in the endocrine system of ascidians.

Large-scale expressed sequence tag (EST) analyses facilitate investigation of the expression and function of genes involved in developmental and physiological processes of this primitive chordate. In collaboration with *Ciona* cDNA consortium members, our laboratory has conducted comprehensive studies of gene expression profiles during embryogenesis (e.g., Satou et al., 2001). In addition, comprehensive gene expression profiles of *C. intestinalis* have been examined in the testis (Inaba et al., 2002) and blood cells (Shida et al., 2003). A previous EST analysis using a subtractive cDNA library of the *Ciona* neural complex presented genes involved in the formation and maintenance of the structures and function of the central nervous system (CNS) (Takamura et al., 2001).

In the present study, we focused on genes that are expressed abundantly in the neural complex and are implicated in the endocrine system of this animal. We report here 21 candidates of genes associated with the endocrine system. In addition, we identified and characterized the *Ciona* prohormone convertase 2 (Ci-PC2) and carboxypeptidase E (Ci-CPE), which serve as processing enzymes associated with endoproteolytic processing of peptide hormone precursors.

2. Materials and methods

2.1. cDNA library construction and EST sequencing

Ciona intestinalis were cultivated at the Maizuru Fisheries Research Station of the Kyoto University, Maizuru, Japan. The neural complex was dissected from adults. The cDNA library was constructed with poly(A)$^+$ RNA isolated from the neural complex, and arrayed in 384-well plates in a Genetix Q-Pix robot. EST sequence was determined by the conventional procedures using the BigDye terminators on ABI3700 autosequencers at the Academia DNA Sequencing Center, National Institute of Genetics, Japan. Details of construction of the cDNA library and EST sequencing procedures have been reported previously (Satou et al., 2003).

2.2. Clustering and similarity search

Using the 3′-most sequence tags, clones were grouped into clusters, each of which contained cDNA clones encoding the same gene, as previously reported (Satou et al., 2001). In order to select clones that were preferentially expressed in the neural complex, we compared the frequency of cDNA clones (EST counts) in the neural complex with those of other adult tissues, including ovary, testis, heart, endostyle, and blood (Satou et al., 2003). The number of cDNA clones of each tissue was normalized by the total number of EST counts. When the EST counts of genes in the neural complex were more than twice that in other tissues, we classed them as being preferentially expressed in the neural complex. Because tag sequences often do not include enough coding sequence to identify their orthology, we aligned these ESTs to gene-models predicted from the draft genome (Dehal et al., 2002) using the BLAST program (BlastN). We identified and used the best-hit gene model as a representative gene sequence. When no corresponding gene-models were found, we used the tag-sequences instead.

NCBI Reference Sequence (RefSeq) records for human proteins (Pruitt et al., 2003), which were released on Aug 24, 2004, were searched with the gene models or ESTs using the BLAST algorithm (BlastX). We next determined the optimum *E*-value score. Cluster ID 03962 (clone ID cilv043o14) has already been annotated with gonadotropin-releasing hormone receptor 3 (GnRHR) (Tello et al., 2005). When a cutoff value of lower than $1.0E-20$ was applied, this cluster showed no similarity to the human protein. It did, however, show similarity to GnRHR, under the cutoff value of $E>-15$. Similar results were obtained in the corresponding clusters of Ci-GnRHR2 and Ci-GnRHR4. On the other hand, Cluster ID 10218, which is annotated with Ci-GnRHR1, showed similarity to GnRHR under the cutoff value of $E>-20$.

Subsequently, we determined that the cutoff value was $1.0E-15$. Where a representative sequence showed low ($E>-15$) or no similarity to human protein, we selected other EST sequences from the same cluster, and searched with these sequences against the same database using BlastX. We categorized the clusters into several groups according to their functions predicted from orthologous human genes (see Section 3).

2.3. Characterization of full-length sequence of Ci-PC2 and Ci-CPE

In this study, we characterized a cDNA clone, cilv052c07, for Ci-PC2. The nucleotide sequence of cDNA insert was determined by standard procedures using BigDye terminators and an ABI PRISM 3100 sequencer (Applied Biosystems).

The sequence of Ci-CPE (ciad075c14, accession number: AK113574) was determined in a previous study (Satou et al., 2002). However, this clone lacked its 5′UTR. We performed 5′-rapid amplification of the cDNA ends (RACE). Total RNA (1 μg) from the neural complex was reverse-transcribed to the template cDNA at 42 °C for 50 min using the oligo-dT anchor primer and SuperScript II reverse transcriptase (Invitrogen). The 5′-RACE was performed using the gene specific primer (5′-CGTTCTTTCGCCCCCTGA-3′) followed by nested PCR with nested primers supplied in the BD SMART RACE cDNA Amplification kit (Clontech) and a gene specific primer (5′-AGGACTTCCCGCCCAACT ACTTCGT-3′). Subcloned inserts were sequenced on an ABI PRISM 3100 sequencer (Applied Biosystems) using BigDye terminators.

2.4. Prediction of domain configuration

Motifs and domains of deduced amino acid sequences were searched with SMART (http://smart.embl-heidelberg.de/).

2.5. Molecular phylogenetic analysis

The amino acid sequences were aligned using the CLUSTAL program (Higgins and Sharp, 1988) and the alignment was checked by hand. After removing gaps, the verified alignments were used to construct phylogenetic trees. The trees were calculated using the MEGA program based on the neighbor-joining method (Saitou and Nei, 1987; Kumar et al., 2001). The sequences used are designated by accession number, abbreviation of the species (HS for *Homo sapiens*, GG for *Gallus gallus*, RC for *Rana catesbeiana*, OL for *Oryzias latipes*, DR for *Danio rerio*, BC for *Branchiostoma californiense*, AC for *Aplysia californica*, CE for *Caenorhabditis elegans*, AM for *Apis mellifera*, HR for *Halocynthia roretzi*, HV for *Hydra vulgaris*, and DM for *D. melanogaster*), and gene name. For example, human proprotein convertase subtilisin/kexin type 2 (PC2) (Accession No. NP_002585) is represented as NP_002585 HS-PC2.

2.6. Whole-mount in situ hybridization

Tunics of young adult specimens for Whole-mount in situ hybridization (WISH) were removed as described previously (Ogasawara et al., 2002). For