Peptidomic Analysis of the Central Nervous System of the Protochordate, *Ciona intestinalis*: Homologs and Prototypes of Vertebrate Peptides and Novel Peptides

Tsuyoshi Kawada, Michio Ogasawara, Toshio Sekiguchi, Masato Aoyama, Kohji Hotta, Kotaro Oka, and Honoo Satake

Suntory Institute for Bioorganic Research (T.K., T.S., M.A., H.S.), Osaka 618-8503, Japan; The Graduate School of Advanced Integration Science (M.O.), Chiba University, Chiba 263-8522, Japan; and Department of Bioscience and Informatics (K.H., K.O.), Faculty of Science and Technology, Keio University, Yokohama 232-8522, Japan

The phylogenetic position of ascidians as the chordate invertebrates closest to vertebrates suggests that they might possess homologs and/or prototypes of vertebrate peptide hormones and neuropeptides as well as ascidian-specific peptides. However, only a small number of peptides have so far been identified in ascidians. In the present study, we have identified various peptides in the ascidian, *Ciona intestinalis*. Mass spectrometry-based peptidomic analysis detected 33 peptides, including 26 novel peptides, from *C. intestinalis*. The ascidian peptides are largely classified into three categories: 1) prototypes and homologs of vertebrate peptides, such as galanin/galanin-like peptide, which have never been identified in any invertebrates; 2) peptides partially homologous with vertebrate peptides, including novel neurotesin-like peptides; 3) novel peptides. These results not only provide evidence that *C. intestinalis* possesses various homologs and prototypes of vertebrate neuropeptides and peptide hormones but also suggest that several of these peptides might have diverged in the ascidian-specific evolutionary lineage. All *Ciona* peptide genes were expressed in the neural complex, whereas several peptide gene transcripts were also distributed in peripheral tissues, including the ovary. Furthermore, a *Ciona* neurotensin-like peptide, *C. intestinalis* neurotensin-like peptide 6, was shown to down-regulate growth of *Ciona* vitellogenic oocytes. These results suggest that the *Ciona* peptides act not only as neuropeptides in the neural tissue but also as hormones in nonneuronal tissues and that ascidians, unlike other invertebrates, such as nematodes, insects, and sea urchins, established an evolutionary origin of the peptidergic neuroendocrine, endocrine, and nervous systems of vertebrates with certain specific molecular diversity. **(*Endocrinology* 152: 2416–2427, 2011)**

Ascidians, invertebrate deuterostome marine animals, belong to the subphylum Tunicata or Urochodata in the phylum Chordata. Their critical phylogenetic position as a protochordate has proven to be highly useful in various fields of the biological sciences as a direct model or ancestor for vertebrates. The establishment of fundamental experimental methods for and biological information on the cosmopolitan species, *Ciona intestinalis*, including the draft genome and expression sequence tags (EST) (1), morpholino DNA gene silencing (2), and Minos transposon-based transgenic technology (3, 4), has led to the postgenomic comprehensive studies, such as the blueprint of the transcriptional regulatory networks in embryogenesis and development of whole chordates (5–7).

In addition to these advantages of *C. intestinalis*, the characterization of *Ciona* peptide hormones and neuropeptides is expected to provide crucial clues to the elucidation of the molecular and functional evolutionary as-

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Abbreviations: Ci-CT, *C. intestinalis* calcitonin; Ci-INS-L, *C. intestinalis* insulin-like peptide; CRF, corticotropin-releasing factor; CT, calcitonin; 3D, three-dimensional; ESI, electrospray ionization; EST, expression sequence tag; GALP, galanin-like peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; INS, insulin; LF, Leu-Phe; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; NT, neurotensin; NTLP, neurotensin-like peptide; Q, quadrupole; t-GnRH, tunicate GnRH; TK, tachykinin; TOF, time of flight; VP, vasopressin; YFV/L, Tyr-Phe-Val/Leu.

pects of endocrine, neuroendocrine, and nervous systems of chordates as well as the biological roles of the *Ciona* neuropeptides and hormones. Moreover, our previous studies verified a novel protease-associated oocyte growth pathway regulated by a neuropeptide, tachykinin (TK), using *C. intestinalis* (8, 9), although the biological role of TK in the ovary of vertebrates still remains to be determined (9). These findings also suggest excellent potential of *C. intestinalis* as a novel model organism for the investigation of endocrinology, neuroendocrinology, and neuroscience in vertebrates.

Several peptides have so far been isolated from ascidians. For instance, cionin, a cholecystokinin/gastrin family peptide, is the first neuropeptide isolated from tunicates, including *C. intestinalis* (10). Moreover, two forms of pituitary adenylate cyclase-activating polypeptide were isolated from another tunicate species, *Chelyosoma productum*, whereas no pituitary adenylate cyclase-activating polypeptide genes have been detected in the genome of *C. intestinalis* (11, 12). To date, several ascidian genes encoding long hormone peptides and/or neuropeptides, including insulin (INS)-like peptides (Ci-INS-L) (13), calcitonin (CT) [*C. intestinalis* calcitonin (Ci-CT)] (14), and corticotropin-releasing factor (CRF) (Ci-CRF) (15), have been detected by homology search of the *Ciona* genome/EST databases owing to conservation of the consensus motifs. In contrast, homology-based search methods are frequently useless for detection of small peptides or their genes, given that major neuropeptides and peptide hormones contain short sequences and that their precursors have slight homology, even though *Ciona* homologs of vertebrate peptide hormones and neuropeptides harbor complete consensus motifs. Additionally, novel peptides or peptide homologs with partial consensus motifs cannot be detected by any form of homology search. Indeed, in the previous study, we identified *Ciona* short peptide homologs, TK (Ci-TK) (8), vasopressin (VP) (Ci-VP) (16), and GnRH-related peptides (Ci-GnRH-X) (17), by mass spectrometry (MS) of the extracts of the central nervous system, whereas these peptides failed to be identified by genomic analyses, including homology search and *in silico* computational prediction of peptide genes.

In this study, we report the MS-based peptidomic characterization of 33 peptides, including 26 novel peptides from the *Ciona* neural complex followed by the detection of their genes by referencing the resultant peptide sequences to the genome/EST database and by localization of their gene expression and the elucidation of biological effects of a novel peptide.

Materials and Methods

Animals

C. intestinalis adults were cultivated and collected at the Maizuru Fisheries Research Station of Kyoto University (Kyoto, Japan) and maintained in sea water at 18 C.

PCR primers

All PCR primers were obtained from Sigma-Aldrich Japan (Tokyo, Japan).

Detection of *Ciona* peptides by MS

Seven-gram *Ciona* neural tissues were pulverized by grinding under liquid nitrogen and extracted in 80 ml of methanol/water/acetic acid solution (90:9:1). The evaporated and concentrated extract was separated by gel filtration column Superdex Peptide 10/300 GL (GE Healthcare, Buckinghamshire, UK), and the eluates were evaporated and concentrated. To separate the peptides more completely, the eluates were treated with ZipTip SCX (Millipore, Billerica, MA). The peptides were eluted by 10 mM, 0.1 M, 0.3 M, 1 M formic ammonium (pH 4.0) stepwise, and the elutes were evaporated and concentrated. The prepared solutions were directly applied to C18 reverse-phase nano/micro column (Capillary Ex-Nano inertsil Peptides C18 0.2 × 150 mm; GL Sciences, Inc., Torrance, CA) with a 1200 Series high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA), and the elutions were spotted to the Prespotted AnchorChip Set for Proteomics (Bruker Daltonics, Bremen, Germany). The peptides on the anchorchip were measured using an ultraflex III, matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF)/TOF instrument (Bruker Daltonics).

Furthermore, the detection of *Ciona* peptides was performed using a MicroMass electrospray ionization (ESI)-quadrupole (Q)-TOF device (Waters, Milford, MA). Peptide extracts were prepared as mentioned above and then applied to a C18 reverse-phase column (COSMOSIL 518-MS-II 4.6 × 150 mm; Nacalai Tesque, Inc., Kyoto, Japan) with LC-10AS high-performance liquid chromatography system (Shimadzu, Kyoto, Japan). Subsequently, the resultant fractions were evaporated and mixed with acetonitrile/formic acid solution. These samples were analyzed using the ESI-Q-TOF instrument.

To detect the peptides bound via disufide bridges, the peptide fraction was subjected to a reduction of the Cys residues by iodacetylation between ZipTip SCX separation and C18 reverse-phase separation. The fraction purified by ZipTip SCX was dissolved with 0.1 M sodium bicarbonate (pH 9.0) and 4.5 mM dithiothreitol and incubated for 30 min at 55 C; 1/10 volume iodacetic acid was added to the peptide solutions, and the mixture was incubated for 15 min at room temperature. Subsequently, the C18 reverse-phase separation and the MALDI analysis were performed as stated above.

The MS/MS data of the peptide ion peaks were comprehensively analyzed with the in-house Mascot server (Matrix Science, Tokyo, Japan) introduced by JGI *C. intestinalis* version 2.0 and/or Kyotograil 2005 gene model databases. The detection program was set to also detect modified peptides: pyroglutamination of the N terminus, amidation of the C terminus, oxidation of Met residues, and iodacetylation of Cys residues.

Identification of the precursors encoding *Ciona* neuropeptides and peptide hormones

Mascot database search detected *Ciona* peptide precursor candidates in the *Ciona* genome/EST database. We identified authentic peptide precursors on the basis of the two following criteria for precursors of neuropeptides and peptide hormones: 1) the putative peptide sequences were flanked by common mono- or dibasic endoproteolytic sites, and 2) the precursors harbored a signal peptide region at their N termini. The presence of a signal peptide region was confirmed by InterProScan Sequence Search (http://www.ebi.ac.uk/Tools/InterProScan/).

Reverse transcription-PCR

Total RNA extracted from the *Ciona* tissues was reverse-transcribed to the template cDNA at 55 C for 60 min using the oligo(dT) anchor primer and the avian myeloblastosis virus reverse transcriptase supplied in the 5′/3′-rapid amplification of the cDNA ends kit (Roche Applied Science, Sandhofer, Germany). RT-PCR for the *Ciona* tissue RNA was performed with 1 μl template cDNA solution, gene specific primers (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), and Taq^{Ex} polymerase (Takara, Kyoto, Japan) using a thermal cycler (model GeneAmp PCR system 9600; Applied Biosystems, Foster City, CA). The PCR program was 94 C for 3 min and 35 cycles of 94 C for 30 sec, 50 C for 30 sec, and 72 C for 30 sec. To confirm the amplified PCR products sequence, the products were sequenced with the primers used for RT-PCR on an ABI Prism 3130/xl Genetic Analyzer (Applied Biosystems) using a Big-Dye sequencing kit (Applied Biosystems).

In situ hybridization

The open reading frame region of each of the peptide cDNA was amplified using neural tissue cDNA and gene-specific primers (Supplemental Table 2). The PCR program was 94 C for 3 min, 35 cycles of 94 C for 30 sec, 50 C for 30 sec, and 72 C for 45 sec and final extension at 72 C for 7 min. The resultant PCR product was inserted into the pCR II TOPO dual promoter vector (Invitrogen, San Diego, CA) according to the manufacturer's instruction and supplied to preparation of RNA probes as a template. Digoxigenin-labeled RNA antisense and sense probe for each *Ciona* peptide gene was prepared using a digoxigenin-labeled RNA labeling kit (Roche Diagnostics, Tokyo, Japan). The neural complexes were dissected and fixed in Bouin's fluid at 4 C overnight. Whole-mount *in situ* hybridization of the neural complex and preparation of 5-μm sections of the hybridized neural complex were performed as previously reported (8, 18, 19). No signals were observed when sense probes were used, confirming the specificity of hybridization.

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, 1 μM Ci-TK, or 1 μM Ci-neurotensin (NT)-like peptide (NTLP)-6 for 15 h as previously described (9). 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) as previously reported (9). In brief, $\Delta\Delta$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 3.

Morphological observation of oocytes

Vitellogenic (stage II) oocytes and postvitellogenic (stage III) oocytes were classified based on morphological characteristics, such as structure of follicle cells, oocyte size, and localization of mitochondria (9, 20, 21). Approximately 20 cells/well of stage II oocytes were isolated from the ovary, transferred into 200 μl of filtered sterile seawater in a 96-well plate, and incubated at 18 C for 15 h after addition of 0.1 μM Ci-TK-I and/or 0.1 μM Ci-NTLP-6. Tripan-blue-based cell staining confirmed viability of more than 90% of the isolated oocytes at each stage during incubation with all reagents under this condition. Each oocyte was fixed using 4% paraformaldehyde and PBS and was incubated with 0.25 μl/ml MitoTracker Red (Molecular Probes, Eugene, OR) and Alexa Fluor 488 phalloidin (Invitrogen) as previously reported (9). Mitochondria localization of each oocyte was observed using a confocal microscopy LSM510 META NLO (Carl Zeiss, Tokyo, Japan). To reconstruct the three-dimensional (3D) images, 70–150 cross-section images (1 μm each) of Alexa Fluor 488 phalloidin-stained oocytes from top to bottom per sample were collected as previously described (9).

Statistical analysis

Results are shown as mean $\pm$ SEM. Data were analyzed by one-way ANOVA with Dunnett error protection. Differences were accepted as significant for $P < 0.05$.

Results

Peptidomic detection of *Ciona* peptides from the neural complex

The methanol extract of the *C. intestinalis* neural complex was purified by gel filtration, cation-ion exchange, and C18 reverse-phase chromatography. The separated fractions were analyzed using the MALDI-TOF MS, and the MS/MS ion peak profiles provided the putative amino acid sequences of *Ciona* neuropeptides and peptide hormones. Moreover, the purified *Ciona* peptide fractions were also measured using ESI-Q-TOF, and neuropeptide and peptide hormone candidates were further detected. Subsequently, the putative peptide sequences were referred on the *C. intestinalis* genome/EST databases (JGI 2.0 gene model and/or Kyotograil 2005 gene model, see Refs. 1, 22), and the precursor sequences encoding putative neuropeptides or peptide hormones were detected. In general, neuropeptide or peptide hormone precursors harbor a hydrophobic signal peptide sequence at the N terminus, and the mature peptide sequences there are flanked by endoproteolytic mono- or dibasic sites (Lys-Lys/Lys-Arg/Arg-Arg/Arg). According to these criteria, we discriminated authentic neuropeptides and peptide hor-

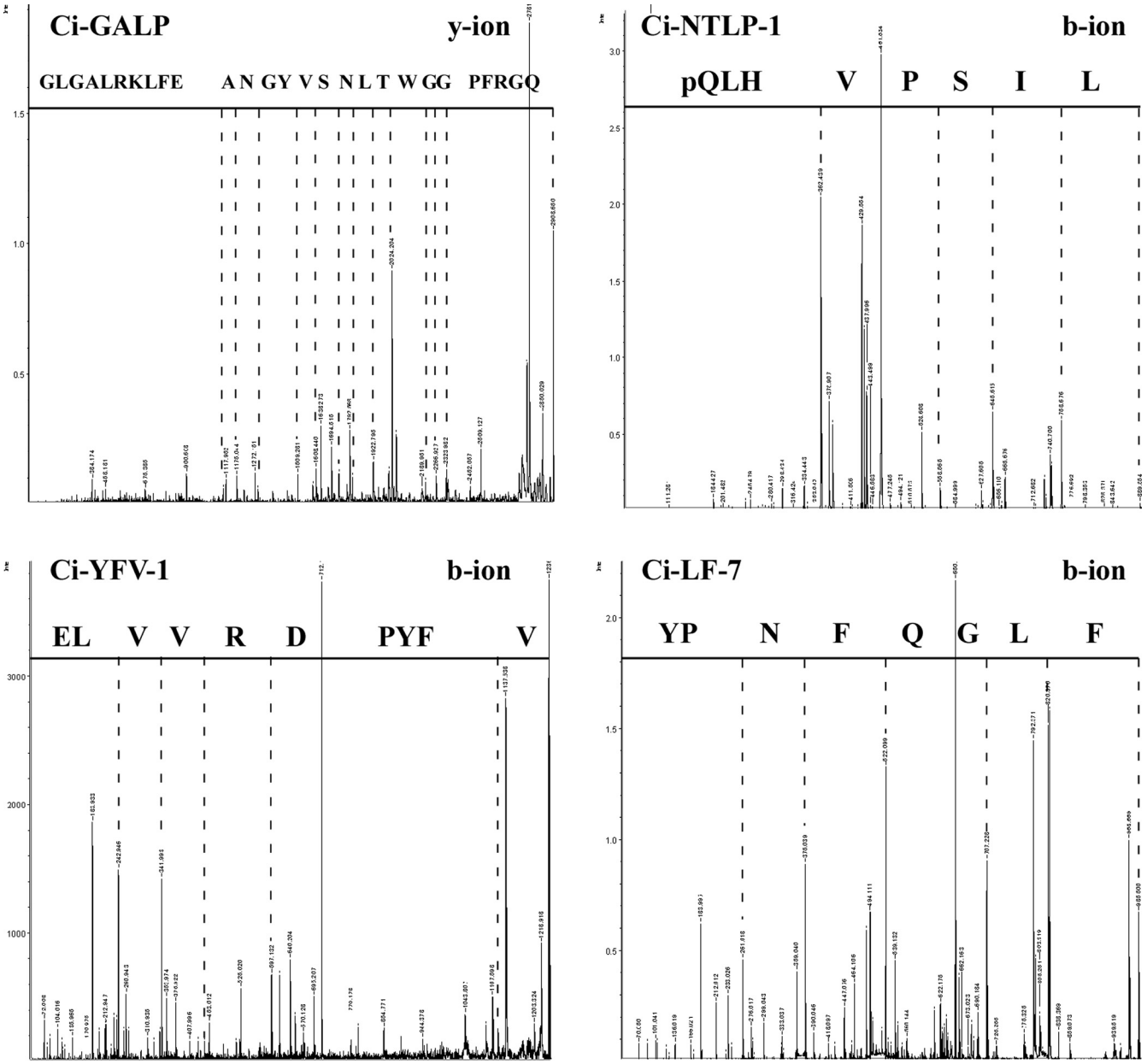

FIG. 1. Representative MS/MS ion peak patterns of *Ciona* peptide sequences. b-Ion is a fragment truncated from N terminus, whereas y-ion loses C-terminal residues. See Supplemental Figs. 1 and 2 for MS/MS ion peak patterns of other *Ciona* peptides.

mones from protein degradation products. Based on such a screening strategy, we eventually characterized 33 peptides encoded in 15 precursors (Fig. 1, Table 1, and Supplemental Figs. 1 and 2). These peptides have molecular weights in the range of 600-3000 (Fig. 1, Table 1, and Supplemental Figs. 1 and 2). In addition, *Ciona* neuropeptides and peptide hormones were approximately 20% of the detected peptide fragments in this study. Notably, Ci-TK, tunicate-GnRH (t-GnRH)-3, t-GnRH-5, t-GnRH-6, Ci-GnRH-X, and Ci-VP, which were identified in the *Ciona* neural complex in previous studies (8, 16, 17, 23), were also detected by this study (Table 1), validating this peptidomic procedure.

Novel *Ciona* homologs and structurally related peptides of vertebrate neuropeptides and peptide hormones

We detected two novel *Ciona* peptide families highly conserved in vertebrates (Fig. 1, Table 1, and Supplemental Fig 1): a galanin/galanin-like peptide (GALP) homolog and NT-related peptides.

Galanin and GALP are vertebrate neuropeptides that have a variety of biological functions (24). The N-terminal region of galanin is critical for its receptor binding and biological function, supported by the fact that the 13-amino acid N-terminal GWTLNSAGYLLGP sequence is completely conserved in galanin of mammals, quail, and

TABLE 1. *Ciona* peptides detected using the MS

Accession no.	Cluster ID	Gene	Peptide	Peptide sequence	Observed MS	Calculated MS
BR000879	00557	*ci-nt-B*	Ci-NTLP-5	NKLLYPSVI	1046.63	1045.62
			Ci-NTLP-6	SRHPKLYFPGIV	1413.79	1412.79
BR000880	03825			AVLHLAINEFQRL	1637.03	1635.95
BR000876	04831	*ci-nt-A*	Ci-NTLP-1	pQLHVPSIL	889.56	888.51
			Ci-NTLP-2	MMLGPGIL	863.45*	862.43*
			Ci-NTLP-3	GMMGPSII	837.44*	836.38*
			Ci-NTLP-4	FGMIPSII	893.50*	892.47*
BR000877	04981	*ci-galp*	Ci-GALP	PFRGQGGWTLNSVGYNAGLGALRKLFE	2908.63	2907.51
BR000881	06757	*ci-lf*	Ci-LF-1	*FQSLF*	*641.19*	*640.33*
			Ci-LF-2	YPGFQGLF	928.56	927.45
			Ci-LF-3	HNPHLPDLF	1089.56	1088.54
			Ci-LF-4	YNSMGLF	831.38	830.36
			Ci-LF-5	SPGMLGLF	821.70	820.42
			Ci-LF-6	SDARLQGLF	1006.54	1005.52
			Ci-LF-7	YPNFQGLF	985.51	984.47
			Ci-LF-8	GNLHSLF	787.42	786.40
BR000882	07684			GFQNNAEGPV	1032.51	1031.47
				SADLFGAPMYII	1513.74	1512.72
AB219239	10761	*ci-gnrh-x*	Ci-GnRH-X	pQHWSNWWIPGAPGYNGa	1851.86	1850.83
AY204706	14373	*ci-gnrh-1*	t-GnRH-3	pQHWSKGYSPGa	1128.55	1127.55
			t-GnRH-5	pQHWSYEFMPGa	1279.54*	1278.51*
			t-GnRH-6	pQHWSYEYMPGa	1295.55*	1294.51*
BR000878	14628			GEKESRPLSSYPGSV	1592.80	1591.78
BR000883	15482			*DPLTNIM*	*803.27*	*802.39*
BR000884	16011			WLRYDA	823.40	822.40
BR000885	32725	*ci-yfv/l*	Ci-YFV-1	ELVVRDPYFV	1236.65	1235.66
			Ci-YFV-2	*NNQESYFV*	*1000.32*	*999.43*
			Ci-YFV-3	DDEPRSYFV	1127.51	1126.49
			Ci-YFL-1	DAARPNYYFL	1229.59	1228.59
AB432887	32977	*ci-vp*	Ci-VP	CFFRDCSNMDWYR	1858.70#	1857.69#
AB175738	36631	*ci-tk*	Ci-TK-I	HVRHFYGLMa	1158.62	1157.59
			Ci-TK-II	SIGDQPSIFNERASFTGLMa	2085.03	2085.01*
BR000886	38516			NLLSLLQHAIETANNAYRSPR	2381.33	2380.26

Peptides represented by *nonitalic* or *italic* characters correspond to the peptide detected using MALDI-TOF/TOF or ESI-Q-TOF machines, respectively. pQ and "a" denote a pyroglutamin residue and the C-terminal amidation. Mass values of peptides containing oxidated Met and iodacetylated Cys residues are indicated by *asterisks* and a *sharp*, respectively.

goldfish (Table 2). On the other hand, GALP has so far been characterized only in mammals. GALP also contains the consensus sequence GWTLNSAGYLLGP in the N-terminal region. However, there are three different features between galanin and GALP sequences (Table 2). First, GALP has a longer sequence than galanin (25). Second, GALP is N-terminally elongated by a PAHRGRG sequence upstream of the consensus sequence, whereas galanin contains no amino acids at this region (25). Third, the C terminus of GALP is amidated, whereas that of galanin is not (25). The peptidomic analysis detected a *Ciona* peptide containing a galanin/GALP-like sequence, parent mass ion peak of which corresponds to 2908.63 (Fig. 1 and Table 2). In addition, the detected sequences are flanked by Lys-Arg at both sides, confirming the mature sequence of this peptide (Supplemental Fig. 3). The detected peptide sequence conserves the galanin/GALP consensus-like sequence, GWTLNSVGYNAGL, whereas it has a truncated sequence compared with galanin and GALP (Table 2). Furthermore, the *Ciona* peptide possesses a PFRGQG se-

TABLE 2. Amino acid sequences of galanin, GALP, and Ci-GALP

Peptide	Sequence
Galanin (quail)	GWTLNSAGYLLGPHAVDNHRSFNDKHGFTa
Galanin (goldfish)	GWTLNSAGYLLGPHAIDSHRSLGDKRGVAa
Galanin (human)	GWTLNSAGYLLGPHAVGNHRSFSDKNGLTSa
GALP (human)	PAHRGRGGWTLNSAGYLLGPVLHLPQMGDQDGKRETALEILDLWKAIDGLPYSHPPQPS
Ci-GALP	PFRGQGGWTLNSVGYNAGLGALRKLFE
	** ** ******* **

The consensus sequences between galanin and GALP are *underlined*. *Asterisks* indicate consensus amino acid residues between GALP and Ci-GALP. In addition, C-terminal amidation is represented by "a."

TABLE 3. Amino acid sequences of Ci-NTLP and neurotensin family peptides

Peptide	Sequence
Neurotensin (rat)	pQLYENKPRRPYIL
Neurotensin (chicken)	pQLHVNKARRPYIL
Neuromedin N (rat)	KIPYIL
LANT 6 (chicken)	KNPYIL
Ci-NTLP-1	pQLHVPSIL
Ci-NTLP-2	GMMGPSII
Ci-NTLP-3	MMLGPGIL
Ci-NTLP-4	FGMIPSII
Ci-NTLP-5	NKLLYPSVI
Ci-NTLP-6	SRHPKLYFPGIV

pQ indicates a pyroglutamin residue.

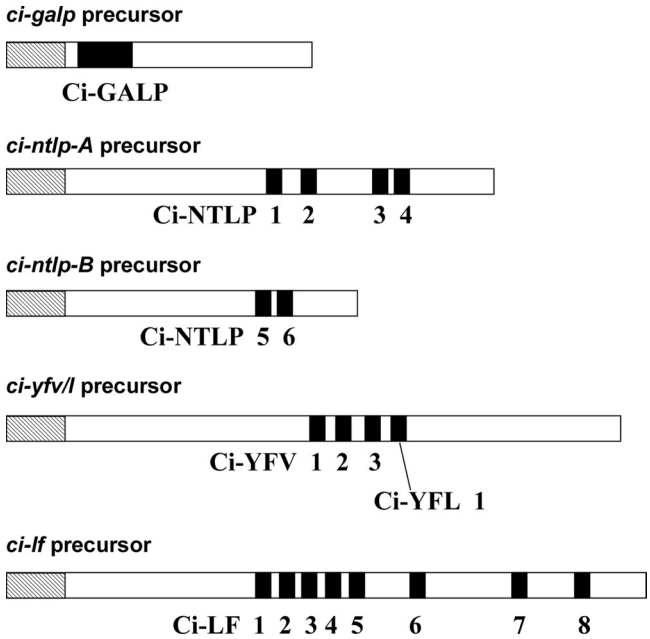

FIG. 2. *Ciona* peptide precursors. Precursors of Ci-GALP, Ci-NTLP, Ci-YFV/L, and Ci-LF are shown. *Closed and slashed squares* show ascidian peptides and putative signal peptides, respectively.

quence at the N terminus, which is homologous with the PAHRGRG sequence in GALP (Table 2). Consequently, we designated the peptide as Ci-GALP. Intriguingly, the C terminus of Ci-GALP, unlike GALP, is not amidated as it is in galanin (Table 2). In addition, no other homologous galanin/GALP-like peptide was found in *C. intestinalis*. These features support the notion that Ci-GALP is a "hybrid peptide" of galanin and GALP. This is the first characterization of galanin/GALP peptides in invertebrates.

Six structurally related peptides were detected by MS, and their parent mass ion peaks corresponded to 889.56, 863.45, 837.44, 893.50, 1046.63, and 1413.79 (Fig. 1, Table 1, and Supplemental Fig. 1). These peptides share a Pro residue at position 4 from the C terminus and two hydrophobic residues (Ile-Leu, Ile-Ile, Val-Ile, and Ile-Val) at the C terminus. These sequences are reminiscent of the C-terminal consensus sequence of NT and their related peptides (Table 3), which are vertebrate neuropeptides responsible for the regulation of various central, gastric, and pituitary functions (26, 27). In addition, the C-terminal consensus motif is crucial for the binding of NT family peptides to their receptors (28, 29). We thus designated these peptides as Ci-NTLP (Table 3). Ci-NTLP are the first invertebrate NT-structurally related peptides. In addition, the *Ciona* gene model search demonstrated that Ci-NTLP were encoded in two precursors; the *ci-ntlp-A* encodes Ci-NTLP-1 to Ci-NTLP-4, whereas the *ci-ntlp-B* encodes Ci-NTLP-5 and Ci-NTLP-6 (Fig. 2 and Supplemental Table 3).

Novel *Ciona* peptides

Nineteen *Ciona* peptides were also detected in the MS analyses (Table 1 and Supplemental Figs. 1 and 2). Two novel peptide groups were characterized in these peptides: Tyr-Phe-Val/Leu (YFV/L) peptide family and Leu-Phe (LF) family. The former includes four novel peptides containing a YFV/L sequence at the C terminus with mass values of 1236.65, 1000.32, 1127.51, and 1229.58, and the latter includes eight novel peptides containing a LF sequence at the C terminus with mass values of 641.19, 928.56, 1089.56, 831.38, 821.70, 1006.54, 985.51, and 787.42 (Fig. 1 and Supplemental Figs. 1 and 2). Accordingly, we designated these peptides as Ci-YFV 1–3, Ci-YFL-1, and Ci-LF 1–8, respectively. The *Ciona* gene model search demonstrated that Ci-YFV/L and Ci-LF are encoded in the individual precursors, respectively (Fig. 2 and Supplemental Fig. 3). Moreover, all Ci-YFV/L and Ci-LF are flanked by endoproteolytic mono- or dibasic site in their precursors (Supplemental Fig. 3). In contrast, other eight novel *Ciona* peptides, unlike Ci-YFV/L and Ci-LF, are encoded by the corresponding precursors as a single peptide flanked by typical basic endoproteolytic sites (Fig. 2 and Supplemental Fig. 3). In addition, these peptides and their precursors display no sequence homology with known neuropeptides.

Tissue distribution patterns of *Ciona* neuropeptide and hormone peptide mRNA

To determine the tissue distribution of *Ciona* peptide mRNA, RT-PCR was performed (Fig. 3 and Supplemental Fig. 4). Figure 3 shows the expression of nine genes: *ci-galp*, *ci-gnrh-1*, *ci-gnrh-x*, *ci-tk*, *ci-vp*, *ci-ntlp-A* and *ci-ntlp-B*, *ci-lf*, and *ci-yfv/l* as stated above. All genes were expressed in the neural complex, and *ci-gnrh-1*, *ci-gnrh-x*, *ci-vp*, *ci-ntlp-B*, and *ci-lf* mRNA were specifically detected in the neural complex. In contrast, *ci-galp*, *ci-tk*, *ci-ntlp-B*, and *ci-yfv/l* were expressed not only in the neural complex but also in other peripheral tissues. *ci-galp* mRNA was also detected in the digestive tract, whereas *ci-ntlp-B*

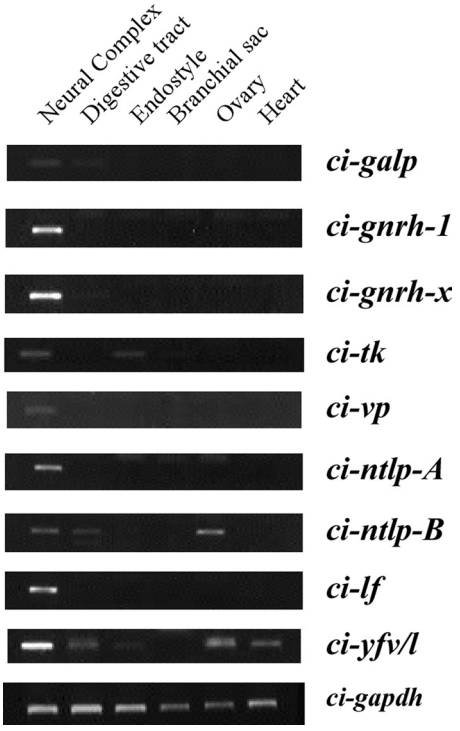

FIG. 3. Tissue distribution of mRNA encoding certain *Ciona* peptides.

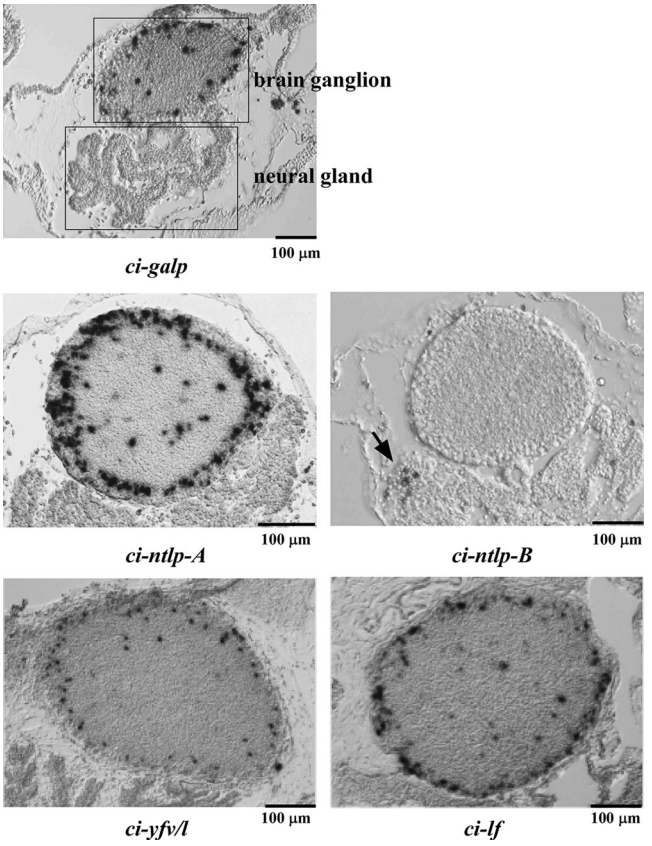

FIG. 4. Expression of peptide genes in the neural complex. An *arrowhead* indicates the *ci-ntlp-B*-positive cells in the neural gland.

mRNA was present in the digestive tract and the ovary (Fig. 3). In addition, *ci-yfv/l* mRNA was distributed in all tissues except the branchial sac. Such gene expression in peripheral tissues suggests that Ci-GALP, Ci-YFV/L, Ci-NTLP-5, and Ci-NTLP-6 also serve as hormonal factors. Moreover, *in situ* hybridization demonstrated that *ci-galp*, *ci-ntlp-A*, *ci-lf*, and *ci-yfv/l* mRNA were localized to various neurons in the brain ganglion (Fig. 4), providing evidence that Ci-GALP, Ci-NTLP-1 to NTLP-4, Ci-LF, and Ci-YFV/L serve as neuropeptides. In contrast, *ci-ntlp-B* was shown to be expressed exclusively in the neural gland (Fig. 4), which is a nonneuronal ovoid body with a sponge texture lying immediately ventral to the brain ganglion (21, 30). Combined with the expression of *ci-ntlp-B* in the ovary (Fig. 3), these data suggest that Ci-NTLP-5 and Ci-NTLP-6 are exclusively nonneural hormonal factors.

Inhibitory effects of Ci-NTLP-6 on oocyte growth

In the previous study, we substantiated that Ci-TK-I enhanced oocyte growth from the vitellogenic stage (stage II) to the postvitellogenic stage (stage III) via up-regulation of the gene expression and enzymatic activities of Ci-cathepsin D, Ci-carboxypeptidase B1, and Ci-chymotrypsin (9). We thus examined the effect of several *Ciona* novel peptides on the protease-associated oocyte growth. As shown in Fig. 5A and Supplemental Table 4, Ci-TK-I induced a prominent elevation of the gene expression of the Ci-cathepsin D (cluster ID no. on the *C. intestinalis* genome/EST, 16624), two subtypes of Ci-carboxypeptidase B1 (16601 and 37777), and two subtypes of Ci-chymotrypsins (2785 and 32073), as previously reported (9). A striking feature is that treatment of the *Ciona* ovary with Ci-NTLP-6 resulted in a marked reduction of the expression of these protease genes, whereas expression of a housekeeping gene, Ci-GAPDH, was not affected (Fig. 5A and Supplemental Table 4); 2785, 16601, 16624, 32073, and 37777 were reduced to approximately 5, 1, 13, 8, and 19%, respectively, compared with the protease gene expression in the untreated ovary (Fig. 5A and Supplemental Table 4). These results indicated the inhibitory regulation of the Ci-TK-I-induced proteases by Ci-NTLP-6.

Whole stage II and stage III oocytes are discriminated by their morphological features and mitochondria localization that were observed by confocal microscopic cross-sections and 3D images (9). Stage II oocytes have 50–70 μm in diameter and a cube-shaped follicle structure, and mitochondria are scattered throughout the cytoplasm (Fig. 5B). At stage III, oocytes have 100-μm diameter and grown follicles with more outstanding and larger petal-like structures, and mitochondria are localized to the oocyte periphery (Fig. 5B). Based on this morphological information, we previously demonstrated that Ci-TK-I specifically enhanced the growth of oocytes from stage II

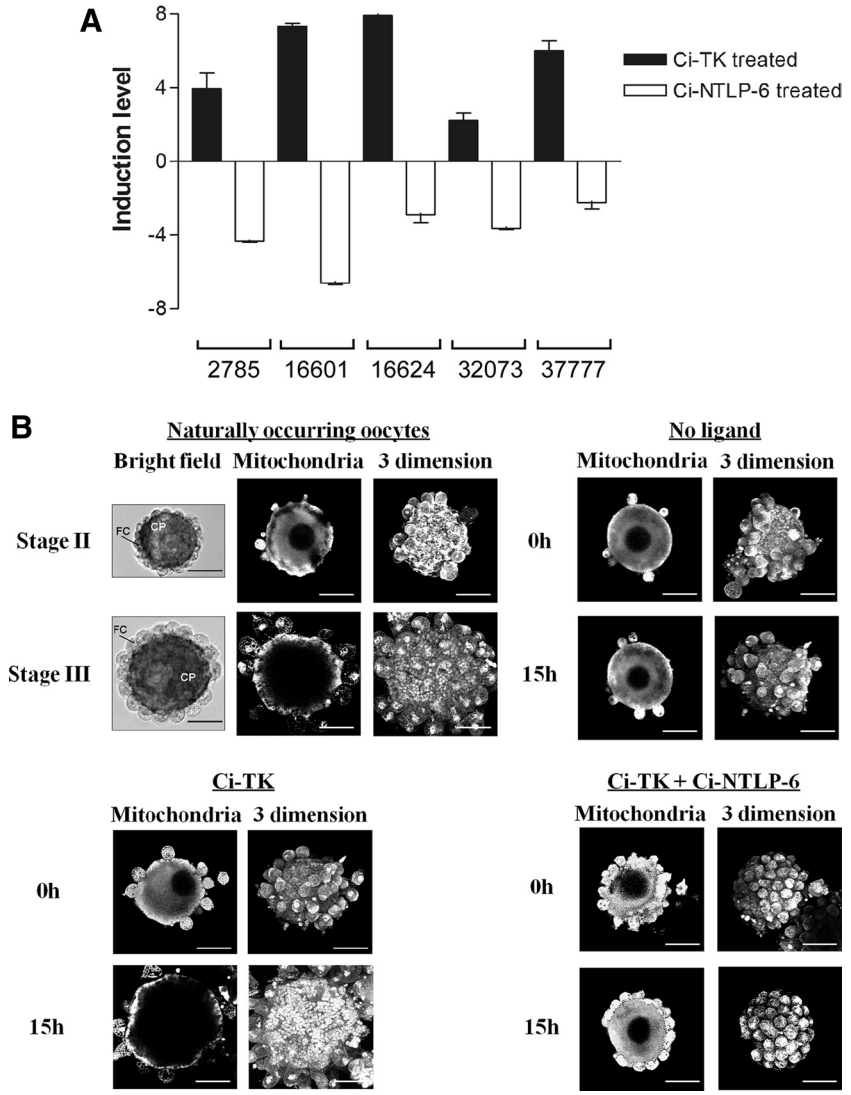

FIG. 5. Effects of Ci-TK-I and Ci-NTLP-6 on oocyte growth. A, Real-time PCR-based quantification of the gene expression of the protease genes in the ovary treated with Ci-TK or Ci-NTLP-6; 2785, 16601, 16624, 32073, and 37777 indicate the cluster ID no. of Ci-chymotrypsin, Ci-carboxypeptidase B1, Ci-cathepsin D, Ci-chymotrypsin subtype, and Ci-carboxypeptidase B1 subtype, respectively, on the *Ciona* genome database (9). The induction level of each gene expression regulated in the ovary treated with Ci-TK-I or Ci-NTLP-6 was calculated from the ΔΔCt values (see Supplemental Table 4). Data are shown as the means of three independent experiments ± SE ($P < 0.05$). B, Optical microscopic images (*left*), the cross-sections of mitochondria localization by fluorescent confocal microscopic observation using MitoTracker Red (*middle*), and 3D (*right*) images of the whole oocytes obtained by reconstruction of 70–150 cross-sections using Alexa Phalloidin 488 (*right*) of a naturally occurring vitellogenic (stage II) and postvitellogenic oocyte (stage III) are shown in the *panel* "naturally occurring oocytes." CP, Cytoplasm of oocytes; FC, follicle; gv, germinal vesicles. Stage II oocytes (50–70 μm in diameter) have a cube-shaped follicle structure surrounding the oocytes, and the mitochondria are abundantly observed throughout the oocyte cytoplasm. Stage III oocytes have grown follicles with more outstanding petal-like structures surrounding 100-μm diameter oocytes, and the fluorescent intensity of mitochondria is markedly localized to the oocyte periphery. No change was observed after incubation in seawater for 15 h ("no ligand" *panel*). A 15-h incubation of stage II oocytes with Ci-TK-I induced stage III oocyte-typical petal-like follicles, mitochondria localization, and size increase (Ci-TK *panel*). However, administration of Ci-NTLP-6 with Ci-TK-I completely blocked the Ci-TK-I-induced growth of stage II oocytes (Ci-TK + Ci-NTLP-6 *panel*).

to stage III (9). In the present study, we evaluated the effect of Ci-NTLP-6 on the Ci-TK-I-induced oocyte growth by the same confocal microscopic observation. As shown in Fig. 5B, the treatment of stage II oocytes with Ci-TK-I and Ci-NTLP-6 resulted in a complete loss of the Ci-TK-I-induced morphological change and mitochondria localization, whereas the morphological change and mitochondria localization were observed in the presence of Ci-TK-I alone as previously reported (9). In addition, no oocyte change was observed in the absence of ligands (Fig. 5B). Collectively, these results indicated that Ci-NTLP-6 inhibited the growth of stage II oocytes via down-regulation of the Ci-TK-I-up-regulated proteases.

Discussion

In the postgenome era, there is increasing importance in the investigation of biological roles of translated gene products, including peptides, not only genetic transcripts. In particular, the identification of neuropeptides and peptide hormones is crucial for understanding the endocrine, neuroendocrine, and nervous systems of organisms. In the past decade, genomic sequences have been determined in various animals, including *C. intestinalis*. A homology-database search is almost useless for the identification of short peptides and novel peptides, although such peptides do nevertheless participate in the regulation of a great variety of biological events. Indeed, only long peptides, *e.g.* INS, CT, and CRF, have been detected by homology searching of the *C. intestinalis* genome/EST database but no short peptides (13–15). Moreover, no *Ciona* peptide gene by computational prediction of peptide genes on the database has been reported. Such a disadvantage hinders progress in molecular and functional characterization of peptides and the evolutionary process of the relevant peptidergic systems. Instead, MS-based peptidomics detected short bioactive peptides of various organisms (31–35). In the present study, we explored 33 short peptides and their genes from the neural complexes of *C. intestinalis* by a combination of MS-based peptido-

mic analyses and a referencing of the resultant sequences in the *C. intestinalis* genome/EST database, followed by localization of the gene expression and the elucidation of the novel inhibitory effect of Ci-NTLP-6 on oocyte growth.

The *Ciona* peptides characterized by our and other groups are largely classified into three categories: 1) prototypes and/or homologues of vertebrate peptides (cionin, t-GnRH, Ci-TK, Ci-CT, Ci-INS-L, Ci-CRF, and Ci-GALP), 2) partial homologs of vertebrate peptides (Ci-GnRH-X, Ci-VP, and Ci-NTLP), and 3) novel peptides, including Ci-LF and Ci-YFV/L. In contrast, no authentic orthologs of TK, CT, GALP, CRF, or NT have so far been identified in any other invertebrates, including *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Strongylocentrotus purpuratus*. These findings provide indisputable evidence that *C. intestinalis* possesses a greater number of homologs to vertebrate peptides compared with other invertebrates. In other words, many peptide prototypes originated from common ancestor chordates of ascidians and vertebrates, not from ancient vertebrates. This view is compatible with the fact that ascidians occupy a phylogenetic position that is closer to vertebrates in evolutionary lineages of animals than any other invertebrates. Of interest is whether these peptide prototypes emerged in other deuterostome invertebrates: hemichordates or cephalochordates. Unfortunately, very few peptides have so far been characterized in hemichordates or cephalochordates, and echinoderms such as sea urchins were found to possess almost no homologs of vertebrate peptides (35–39). Peptidomics of the nervous and endocrine systems of cephalochordates followed by a referencing of the resulting sequences to their genome database are expected to provide fruitful insight into the origins and evolutionary processes of deuterostome neuropeptides and peptide hormones.

Also of interest is that novel *Ciona* peptides, including Ci-YFV/L and Ci-LF, were found from the neural complex (Fig. 3 and Table 1). All of their precursors show the characteristics of peptide precursors: the presence of an N-terminal signal peptide region and endoproteolytic sites adjacent to the peptide sequences (Supplemental Fig. 3). The presence of these peptides can be interpreted in three ways. First, these peptides may be novel homologs of unknown vertebrate peptides. If this is true, the functional characterization of these peptides will contribute a great deal to the investigation of novel peptidergic systems in vertebrates. Second, these peptides might have been lost during the evolution of ancestral protochordates to vertebrates. In this case, our present data will provide fruitful insight into the evolutionary processes of peptidergic systems throughout deuterostomes, if nonascidian deuterostome invertebrates, such as cephalochordates and hemichordates, conserve the homologs. Third, they are ascidian-specific peptides. This presumption leads to the unexpected diversity of neuropeptides and/or peptide hormones in chordates. To address these issues, we are now attempting the surveys of genome sequences of vertebrates and invertebrate deuterostomes by computational prediction of peptide genes as well as homology search using the sequences of Ci-YFV/L and Ci-LF. Furthermore, in any cases, these peptides are likely to be involved in biological events in *C. intestinalis*. Functional analysis of these peptides is also currently in progress.

In vertebrates, typical peptide precursors, with several exceptions of enkephalin precursors and TRH precursors (40, 41), encode one or two structurally and functionally related peptides. In contrast, many types of protostome neuropeptide precursors encode multiple copies of structurally related peptides, *e.g.* molluscan and insect FMRFamide peptides (42, 43). In *C. intestinalis*, the precursors of Ci-GALP, Ci-VP, Ci-CT, cionin, Ci-GnRH-X, Ci-TK, and Ci-CRF, like the vertebrate homologs, encode one or two peptides, whereas the precursors of the novel peptides, Ci-YFV/L and Ci-LF, encode multiple copies of structurally related peptides (Fig. 2), which is reminiscent of protostome neuropeptide precursors. Such structural organization of peptide precursors indicates that *Ciona* peptide precursors are classified into "vertebrate type" and "protostome type," which is consistent with the phylogenetic position of ascidians as chordate invertebrates closest to vertebrates.

Of particular significance is that Ci-GALP and Ci-NTLP are the first invertebrate galanin/GALP family peptide and NT-structurally related peptides, respectively. Intriguingly, Ci-GALP displays a hybrid peptide sequence of galanin and GALP (Table 2). Similar hybrid sequences of vertebrate family peptides were previously found in cionin and Ci-CT: cionin contains two sulfated tyrosines at the corresponding positions of vertebrate cholecystokinin and gastrin, respectively (10), and Ci-CT shares typical amino acid residues of CT and CT-gene related peptides (14). These findings support the view that vertebrate galanin and GALP diverged from a Ci-GALP-like ancestor, although GALP has yet to be found in lower vertebrates (44–46). Ci-NTLP, like vertebrate NT, conserve a Pro residue at position 4 from the C terminus and two hydrophobic residues at the C terminus, respectively (Table 3). These data indicate *Ciona*-specific sequence divergence in peptides that are highly conserved in vertebrates. Such *Ciona*-specific sequence divergence or "partial conservation" is found in other *Ciona* peptides; for instance, Ci-GnRH-X and Ci-VP. All chordate GnRH family peptides consist of 10 residues and share the pyroGlu-His-Trp-Ser consensus at the N terminus and Pro-Gly-amide at the C

terminus. Ci-GnRH-X is five amino acid longer than GnRH, and no Pro residue is located at position 2 from the C terminus, although the pyroGlu-His-Trp-Ser and Gly-amide sequences are conserved in Ci-GnRH-X (17). Oxytocin/VP family peptides are nine-residue peptides that harbor a disulfide-bridged N-terminal region, and the C terminus is amidated (47). Ci-VP displays high sequence homology in the N-terminal circular structure, but the C terminus is elongated and not amidated (16). Furthermore, several *Ciona* peptide precursors show unique structural organization. *Ci-ntlp*-A encodes four NT-like peptides (Fig. 2), compared with vertebrate NT precursors in which a single NT and another NT-related peptide, neuromedin N, are present (48, 49). Similar *Ciona*-unique multiple copies of related peptides are seen in *Ciona* GnRH. The vertebrate GnRH I and II are encoded as a single copy in the precursors (50, 51), whereas *ci-gnrh*-1 and *ci-gnrh*-2 encode t-GnRH-3, t-GnRH-5, t-GnRH-6 and t-GnRH-4, t-GnRH-7, t-GnRH-8, respectively (23). These findings indicate that the *Ciona* GnRH family peptides and NTLP might have been generated via *Ciona*-specific paralogous gene duplication and multiplication of peptide sequences in the evolutionary lineage of ascidians. Altogether, the *Ciona* peptides and their precursors show not only common features among chordates but also ascidian-specific diversity in sequences and/or structural organization.

Notably, *Ciona* homologs of vertebrate neuropeptides and/or peptide hormones from various glands, including the hypothalamus, have been identified, but no homologs of vertebrate pituitary hormones, such as ACTH, TSH, FSH, LH, GH, and prolactin, have ever been reported. This is consistent with the fact that ascidians are not endowed with an organ corresponding to a pituitary (21). In addition, ascidians, unlike vertebrates, lack a complete circulation system, suggesting that peptide hormones cannot be transported to target tissues via blood flow in the endocrine system. Collectively, these findings reinforce the evolutionary scenario that the hypothalamus-pituitary endocrine system might have been established in concert with the acquisition of the closed circulation system in the evolutionary process of chordate invertebrates to vertebrates. Moreover, *in situ* hybridization of the *Ciona* neural complex demonstrated that most ascidian peptide mRNA were distributed in neurons of the brain ganglion (Fig. 4). These results lead to the conclusion that most peptides are directly transported to target tissues through nerve fibers in ascidians. In addition, *ci-ntlp B*, like *ci-ct* (14), was shown to be expressed in the neural gland (Fig. 4), a nonneuronal tissue, suggesting that Ci-NTLP-5 and Ci-NTLP-6 are hormonal factors. Furthermore, the expression of *ci-yfv/l* was detected not only in neurons (Figs. 3 and 4) but also in nonneuronal tissues (Fig. 3), similar to the expression of *ci-tk* in neurons and the peripheral tissues (8), suggesting that Ci-YFV/L serve as both neuropeptides and hormones. These results indicate the possibility that paracrine-like local endocrine systems have been established in ascidians. In other words, such a local endocrine system might have emerged in chordate ancestors and subsequently evolved into the advanced endocrine system along with the closed circulation system.

It is noteworthy that a *Ciona* novel peptide, Ci-NTLP-6, elicited a novel inhibitory effect on the Ci-TK-I-induced oocyte growth via down-regulation of Ci-cathepsin D, Ci-carboxypeptidase B1, and Ci-chymotrypsin (Fig. 5). This is the first functional characterization of NT and their related peptides in ovarian functions. Furthermore, 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 (52), the proteolytic production of several component proteins for zona pellucida by carboxypeptidase B1 in mammalian oocytes at an early growth stage (53) and unknown mechanism of chymotrypsin activity in several invertebrates (54, 55). Combined with these findings, the present study suggests that TK and NT elicit a positive and negative activity, respectively, on the protease-associated oocyte growth pathway highly conserved in a wide range of animal species. The regulatory mechanism of the oocyte growth involving Ci-NTLP-6 and Ci-TK-I is now being investigated in greater detail.

The adult ascidian lifestyle mainly consists of reproduction and feeding, suggesting that major ascidian neuropeptides and peptide hormones are in particular responsible for the relevant physiological functions. Moreover, combined with the phylogenetic position of ascidians as protochordates, the elucidation of physiological functions of various *Ciona* homologs of vertebrate neuropeptides and peptide hormones is expected to highlight the applicability of *C. intestinalis* as an unprecedented model organism for the investigation of novel molecular mechanisms underlying reproduction and feeding in vertebrates. To verify the functions of the novel peptides, identification of their receptors is of particular importance, given that the receptors are localized in the target tissues of peptides. Molecular characterization of the receptors of the *Ciona* peptides identified in the present study is currently in progress.

In conclusion, we have characterized novel peptides of *C. intestinalis* using a peptidomic approach. Our present data reveal *Ciona* homologs and/or prototypes of vertebrate neuropeptides and peptide hormones and also certain novel peptides, leading to new insight into their mo-

lecular and functional evolution and diversity throughout chordates and providing a promising model organism for investigating the endocrine, neuroendocrine, and nervous systems of vertebrates.

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Address all correspondence and requests for reprints to: Honoo Satake, Suntory Institute for Bioorganic Research, 1-1-1 Wakayamadai, Shimamoto, Mishima, Osaka 618-8503, Japan. E-mail: satake@sunbor.or.jp.

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References

1. **Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A, Davidson B, Di Gregorio A, Gelpke M, Goodstein DM, Harafuji N, Hastings KE, Ho I, Hotta K, Huang W, Kawashima T, Lemaire P, Martinez D, Meinertzhagen IA, Necula S, Nonaka M, Putnam N, Rash S, Saiga H, Satake M, Terry A, Yamada L, Wang HG, Awazu S, Azumi K, Boore J, Branno M, Chin-Bow S, DeSantis R, Doyle S, Francino P, Keys DN, Haga S, Hayashi H, Hino K, Imai KS, Inaba K, Kano S, Kobayashi K, Kobayashi M, Lee BI, Makabe KW, Manohar C, Matassi G, Medina M, Mochizuki Y, Mount S, Morishita T, Miura S, Nakayama A, Nishizaka S, Nomoto H, Ohta F, Oishi K, Rigoutsos I, Sano M, Sasaki A, Sasakura Y, Shoguchi E, Shin-i T, Spagnuolo A, Stainier D, Suzuki MM, Tassy O, Takatori N, Tokuoka M, Yagi K, Yoshizaki F, Wada S, Zhang C, Hyatt PD, Larimer F, Detter C, Doggett N, Glavina T, Hawkins T, Richardson P, Lucas S, Kohara Y, Levine M, Satoh N, Rokhsar DS** 2002 The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science 298: 2157–2167
2. **Satou Y, Imai KS, Satoh N** 2001 Action of morpholinos in *Ciona* embryos. Genesis 30:103–106
3. **Sasakura Y, Awazu S, Chiba S, Satoh N** 2003 Germ-line transgenesis of the Tc1/mariner superfamily transposon *Minos* in *Ciona intestinalis*. Proc Natl Acad Sci USA 100:7726–7730
4. **Sasakura Y, Nakashima K, Awazu S, Matsuoka T, Nakayama A, Azuma J, Satoh N** 2005 Transposon-mediated insertional mutagenesis revealed the functions of animal cellulose synthase in the ascidian *Ciona intestinalis*. Proc Natl Acad Sci USA 102:15134–15139
5. **Imai KS, Levine M, Satoh N, Satou Y** 2006 Regulatory blueprint for a chordate embryo. Science 312:1183–1187
6. **Kubo A, Suzuki N, Yuan X, Nakai K, Satoh N, Imai KS, Satou Y** 2010 Genomic cis-regulatory networks in the early *Ciona intestinalis* embryo. Development 137:1613–1623
7. **Tassy O, Dauga D, Daian F, Sobral D, Robin F, Khoueiry P, Salgado D, Fox V, Caillol D, Schiappa R, Laporte B, Rios A, Luxardi G, Kusakabe T, Joly JS, Darras S, Christiaen L, Contensin M, Auger H, Lamy C, Hudson C, Rothbächer U, Gilchrist MJ, Makabe KW, Hotta K, Fujiwara S, Satoh N, Satou Y, Lemaire P** 2010 The ANISEED database: digital representation, formalization, and elucidation of a chordate developmental program. Genome Res 20: 1459–1468
8. **Satake H, Ogasawara M, Kawada T, Masuda K, Aoyama M, Minakata H, Chiba T, Metoki H, Satou Y, Satoh N** 2004 Tachykinin and tachykinin receptor of an ascidian, *Ciona intestinalis*: evolutionary origin of the vertebrate tachykinin family. J Biol Chem 279:53798–53805
9. **Aoyama M, Kawada T, Fujie M, Hotta K, Sakai T, Sekiguchi T, Oka K, Satoh N, Satake H** 2008 A novel biological role of tachykinins as an upregulator of oocyte growth: identification of an evolutionary origin of tachykinergic functions in the ovary of the ascidian, *Ciona intestinalis*. Endocrinology 149:4346–4356
10. **Johnsen AH, Rehfeld JF** 1990 Cionin: a disulfotyrosyl hybrid of cholecystokinin and gastrin from the neural ganglion of the protochordate *Ciona intestinalis*. J Biol Chem 265:3054–3058
11. **Sherwood NM, Adams BA, Tello JA** 2004 Endocrinology of protochordates. Can J Zool 83:225–255
12. **Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, Fournier A, Chow BK, Hashimoto H, Galas L, Vaudry H** 2009 Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. Pharmacol Rev 61:283–357
13. **Olinski RP, Lundin LG, Hallböök F** 2006 Conserved synteny between the *Ciona* genome and human paralogons identifies large duplication events in the molecular evolution of the insulin-relaxin gene family. Mol Biol Evol 23:10–22
14. **Sekiguchi T, Suzuki N, Fujiwara N, Aoyama M, Kawada T, Sugase K, Murata Y, Sasayama Y, Ogasawara M, Satake H** 2009 Calcitonin in a protochordate, *Ciona intestinalis*: the prototype of the vertebrate calcitonin/calcitonin gene related peptide superfamily. FEBS J 276:4437–4447
15. **Lovejoy DA, Barsyte-Lovejoy D** 2010 Characterization of a corticotropin-releasing factor (CRF)/diuretic hormone-like peptide from tunicates: insight into the origins of the vertebrate CRF family. Gen Comp Endocrinol 165:330–336
16. **Kawada T, Sekiguchi T, Itoh Y, Ogasawara M, Satake H** 2008 Characterization of a novel vasopressin/oxytocin superfamily peptide and its receptor from an ascidian, *Ciona intestinalis*. Peptides 29:1672–1678
17. **Kawada T, Aoyama M, Okada I, Sakai T, Sekiguchi T, Ogasawara M, Satake H** 2009 A novel inhibitory gonadotropin-releasing hormone-related neuropeptide in the ascidian, *Ciona intestinalis*. Peptides 30:2200–2205
18. **Ogasawara M, Satoh N** 1998 Isolation and characterization of endostyle-specific genes in the ascidian *Ciona intestinalis*. Biol Bull 195:60–69
19. **Ogasawara M, Sasaki A, Metoki H, Shin-i T, Kohara Y, Satoh N, Satou Y** 2002 Gene expression profiles in young adult *Ciona intestinalis*. Dev Genes Evol 212:173–185
20. **Prodon F, Chenevert J, Sardet C** 2006 Establishment of animal-vegetal polarity during maturation in ascidian oocytes. Dev Biol 290:297–311
21. **Burighel P, Cloney RA** 1997 Urochordata: Ascidiacea. In: Harrison FW, Ruppert EE, eds. Microscopic anatomy of invertebrates. Vol 15. Hemichordata, chaetognatha, and the invertebrate chordates. Hoboken, NJ: Wiley; 221–347
22. **Satou Y, Kawashima T, Shoguchi E, Nakayama A, Satoh N** 2005 An integrated database of the ascidian, *Ciona intestinalis*: towards functional genomics. Zoolog Sci 22:837–843
23. **Adams BA, Tello JA, Erchegyi J, Warby C, Hong DJ, Akinsanya KO, Mackie GO, Vale W, Rivier JE, Sherwood NM** 2003 Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*. Endocrinology 144:1907–1919
24. **Lang R, Gundlach AL, Kofler B** 2007 The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. Pharmacol Ther 115:177–207

25. **Ohtaki T, Kumano S, Ishibashi Y, Ogi K, Matsui H, Harada M, Kitada C, Kurokawa T, Onda H, Fujino M** 1999 Isolation and cDNA cloning of a novel galanin-like peptide (GALP) from porcine hypothalamus. J Biol Chem 274:37041–37045
26. **Pothoulakis C, Castagliuolo I, Leeman SE** 1998 Neuroimmune mechanisms of intestinal responses to stress. Role of corticotropin-releasing factor and neurotensin. Ann NY Acad Sci 840:635–648
27. **Tyler-McMahon BM, Boules M, Richelson E** 2000 Neurotensin: peptide for the next millennium. Regul Pept 93:125–136
28. **García-Garayoa E, Allemann-Tannahill L, Bläuenstein P, Willmann M, Carrel-Rémy N, Tourwé D, Iterbeke K, Conrath P, Schubiger PA** 2001 In vitro and in vivo evaluation of new radiolabeled neurotensin(8–13) analogues with high affinity for NT1 receptors. Nucl Med Biol 28:75–84
29. **Kitabgi P** 2006 Functional domains of the subtype 1 neurotensin receptor (NTS1). Peptides 27:2461–2468
30. **Deyts C, Casane D, Vernier P, Bourrat F, Joly JS** 2006 Morphological and gene expression similarities suggest that the ascidian neural gland may be osmoregulatory and homologous to vertebrate periventricular organs. Eur J Neurosci 24:2299–2308
31. **Baggerman G, Cerstiaens A, De Loof A, Schoofs L** 2002 Peptidomics of the larval *Drosophila melanogaster* central nervous system. J Biol Chem 277:40368–40374
32. **Hummon AB, Amare A, Sweedler JV** 2006 Discovering new invertebrate neuropeptides using mass spectrometry. Mass Spectrom Rev 25:77–98
33. **Weaver RJ, Audsley N** 2008 Neuropeptides of the beetle, *Tenebrio molitor* identified using MALDI-TOF mass spectrometry and deduced sequences from the *Tribolium castaneum* genome. Peptides 29:168–178
34. **Menschaert G, Vandekerckhove TT, Baggerman G, Landuyt B, Sweedler JV, Schoofs L, Luyten W, Van Criekinge W** 2010 A hybrid, de novo based, genome-wide database search approach applied to the sea urchin neuropeptidome. J Proteome Res 9:990–996
35. **Xie F, London SE, Southey BR, Annangudi SP, Amare A, Rodriguez-Zas SL, Clayton DF, Sweedler JV** 2010 The zebra finch neuropeptidome: prediction, detection and expression. BMC Biol 8:28
36. **Elphick MR, Thorndyke MC** 2005 Molecular characterisation of SALMFamide neuropeptides in sea urchins. Exp Biol 208:4273–4282
37. **Elphick MR, Rowe ML** 2009 NGFFFamide and echinotocin: structurally unrelated myoactive neuropeptides derived from neurophysin-containing precursors in sea urchins. J Exp Biol 212:1067–1077
38. **Gwee PC, Tay BH, Brenner S, Venkatesh B** 2009 Characterization of the neurohypophysial hormone gene loci in elephant shark and the Japanese lamprey: origin of the vertebrate neurohypophysial hormone genes. BMC Evol Biol 9:47
39. **Mita M, Yoshikuni M, Ohno K, Shibata Y, Paul-Prasanth B, Pitchayawasin S, Isobe M, Nagahama Y** 2009 A relaxin-like peptide purified from radial nerves induces oocyte maturation and ovulation in the starfish, *Asterina pectinifera*. Proc Natl Acad Sci USA 106:9507–9512
40. **Comb MJ, Kobierski L, Chu HM, Tan Y, Borsook D, Herrup K, Hyman SE** 1992 Regulation of opioid gene expression: a model to understand neural plasticity. NIDA Res Monogr 126:98–112
41. **Galas L, Raoult E, Tonon MC, Okada R, Jenks BG, Castaño JP, Kikuyama S, Malagon M, Roubos EW, Vaudry H** 2009 TRH acts as a multifunctional hypophysiotropic factor in vertebrates. Gen Comp Endocrinol 164:40–50
42. **Morishita F, Furukawa Y** 2006 Neuropeptides in *Aplysia*: key players for the neural regulation of physiological functions and behavior. In: Satake H, ed. Invertebrate neuropeptides and hormones: basic knowledge and recent advances. Kerala, India: Transworld Research Network; 37–74
43. **Johnson EC** 2006 Post-genomic approaches to resolve neuropeptide signaling. In: Satake H, ed. Invertebrate neuropeptides and hormones: basic knowledge and recent advances. Kerala, India: Transworld Research Network; 179–224
44. **Kohchi C, Tsutsui K** 2000 Avian galanin: cloning of complementary DNAs and characterization of transcripts in different tissues. J Exp Zool 287:183–190
45. **Unniappan S, Lin X, Peter RE** 2003 Characterization of complementary deoxyribonucleic acids encoding preprogalanin and its alternative splice variants in the goldfish. Mol Cell Endocrinol 200:177–187
46. **Yu JY, Pon CH, Ku HC, Wang CT, Kao YH** 2007 A preprogalanin cDNA from the turtle pituitary and regulation of its gene expression. Am J Physiol Regul Integr Comp Physiol 292:R1649–R656
47. **Kawada T, Sekiguchi T, Sugase K, Satake, H** 2009 Evolutionary aspects of molecular forms and biological functions of oxytocin family peptides In: Jastrow H, Feuerbach D, eds. Handbook of oxytocin research: synthesis, storage and release, actions, and drug forms. Hauppauge, NY: Nova Science Publishers, Inc.; 59–85
48. **Kislauskis E, Bullock B, McNeil S, Dobner PR** 1988 The rat gene encoding neurotensin and neuromedin N. Structure, tissue-specific expression, and evolution of exon sequences. J Biol Chem 263:4963–4968
49. **Vincent JP, Mazella J, Kitabgi P** 1999 Neurotensin and neurotensin receptors. Trends Pharmacol Sci 20:302–309
50. **Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR** 2004 Gonadotropin-releasing hormone receptors. Endocr Rev 25:235–275
51. **Millar RP** 2005 GnRHs and GnRH receptors. Anim Reprod Sci 88:5–28
52. **Carnevali O, Cionna C, Tosti L, Lubzens E, Maradonna F** 2006 Role of cathepsins in ovarian follicle growth and maturation. Gen Comp Endocrinol 146:195–203
53. **Litscher ES, Qi H, Wassarman PM** 1999 Mouse zona pellucida glycoproteins mZP2 and mZP3 undergo carboxy-terminal proteolytic processing in growing oocytes. Biochemistry 38:12280–12287
54. **Sawada-Takagi M, Someno T, Hoshi M, Sawada H** 1989 Inhibition of starfish oocyte maturation by leupeptin analogs, potent trypsin inhibitors. Dev Biol 133:609–612
55. **Tanaka E, Sawada-Takagi M, Sawada H** 2000 Enzymatic properties of the proteasome purified from starfish oocytes and its catalytic subunits involved in oocyte maturation. Comp Biochem Physiol C Toxicol Pharmacol 125:215–223