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Characterization of a novel vasopressin/oxytocin superfamily peptide and its receptor from an ascidian, *Ciona intestinalis*☆

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ABSTRACT

The vasopressin (VP)/oxytocin (OT) superfamily peptides are one of the most widely distributed neuropeptides and/or neurohypophysial hormones, but have ever not been characterized from any deuterostome invertebrates including protochordates, ascidians. In the present study, we show the identification of a novel VP/OT superfamily peptide and its receptor in the ascidian, *Ciona intestinalis*. Intriguingly, the Ciona VP/OT-related peptide (Ci-VP), unlike other 9-amino acid and C-terminally amidated VP/OT superfamily peptides, consists of 13 amino acids and lacks a C-terminal amidation. Mass spectrometry confirmed the presence of the 13-residue Ci-VP in the neural complex. Furthermore, 10 of 14 cysteines are conserved in the neurophysin domain, compared with other VP/OT counterparts. These results revealed that the VP/OT superfamily is conserved in ascidians, but the Ci-VP gene encodes an unprecedented VP/OT-related peptide and neurophysin protein. Ci-VP was also shown to activate its endogenous receptor, Ci-VP-R, at physiological concentrations, confirming the functionality of Ci-VP as an endogenous ligand. The Ci-VP gene was expressed exclusively in neurons of the brain, whereas the Ci-TK-R mRNA was distributed in various tissues including the neural complex, alimentary tract, gonad, and heart. These expression profiles suggest that Ci-VP, like other VP/OT superfamily peptides, serves as a multifunctional neuropeptides. Altogether, our data revealed both evolutionary conservation and specific divergence of the VP/OT superfamily in protochordates. This is the first molecular characterization of a VP/OT superfamily peptide and its cognate receptor from not only ascidians but also deuterostome invertebrates.

1. Introduction

Vasopressin (VP) and oxytocin (OT) are homologous vertebrate neurohypophysial peptide hormones, and both family peptides are conserved in vertebrates [1,8,24]. In mammals, the former participates mainly in osmoregulation, whereas the latter is responsible for reproductive behavior [1,8,24]. The vertebrate VP/OT family precursors are composed of major three regions: a signal peptide, a VP/OT sequence, a neurophysin domain featured by 14 cysteines [1,8,24]. These homologies suggest that the VP and OT family genes separated from the common ancestral gene via gene duplication during

☆ The nucleotide sequences reported in this paper have been submitted to the GenBank™/EMBL/DDBJ Data Bank with accession numbers AB432887 and AB432888.

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Abbreviations: Ci-VP, Ciona vasopressin-related peptide; Ci-VP-R, Ci-VP receptor; GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptor; nt, nucleotide; OT, oxytocin; VP, vasopressin.

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Table 1 – Amino acid sequences of OT/VP family peptides

Peptide	Sequence
Ci-VP	CFFRDCSNMDWYR
VP	CYFQNCPRGa
VT	CYIQNCPRGa
OT	CYIQNCPLGa
MT	CYIQNCPIGa
IT	CYISNCPIGa
annetocin	CFVRNCPTGa
Lys-conopressin	CFIRNCPKGa

Shadowed amino acids are highly conserved in Ci-VP and other vasopressin/oxytocin superfamily peptides. In additon, 'a' denotes C-terminal amide.

evolution of vertebrates. The VP/OT superfamily peptides have also been isolated from protostomes [8,19,20,25,27]. The protostome peptides all share Cys^1, Asn^5, Cys^6, Pro^7, Gly^9, and C-terminal amidation with the vertebrate VP/OT counterparts (Table 1). Similarly, the protostome VP/OT superfamily precursors are all organized by a signal peptide, peptide sequence, and 14-cysteine neurophysin domain [8,19,20,25,27], indicating that the VP/OT superfamily genes are conserved in protostomes. In addition, only one VP/OT superfamily peptide is present in the lowest vertebrate cyclostomes and most protostomes [1,19,20,24,25,27], suggesting that a duplication of the ancestral VP/OT gene might have occurred after the evolutionary process of the Agenta [1,8,24].

The VP/OT superfamily peptides manifest their activities through their receptors, which belong to a G protein-coupled receptor (GPCR) superfamily. To date, three VP receptors (V1aR, V1bR, and V2R) and one OT receptor (OTR) have been identified in mammals [3,7,25]. VP/OT superfamily peptide receptors have also been characterized from several protostomes [11–14,28]. All mammalian and protostome VP/OT superfamily peptide receptors have been shown to trigger an increase in the intracellular calcium ion, except V2R that induces the production of cAMP [7,11–14,26,28].

Despite intensive efforts, no VP/OT superfamily peptides have ever been characterized from deuterostome invertebrates including protochordates, namely, ascidians. The cosmopolitan ascidian species, *Ciona intestinalis*, has been recognized as an excellent model organism in developmental biology and evolutionary biology owing to the critical phylogenetic position as a basal chordate [3,22,23]. Recently, ascidians have been shown to possess orthologs or prototypes for vertebrate neuropeptides and/or hypothalamic hormones, including gonadtropin-releasing hormones (GnRHs), tachykinins, and relaxins/insulins, some of which have not ever been identified in traditional protostome model organisms, e.g., the fruitfly *Drosophila melanogaster* and the nematoda *Caenorhabditis elegans* [2,17,21–23]. These findings suggest that the molecular characterization of VP/OT superfamily peptides from *C. intestinalis* is expected not only to provide crucial clues to the understanding of biological roles of VP/OT superfamily peptides in protochordates and the evolutionary lineages of the VP/OT superfamily throughout chordates, but also to establish a new model organism for investigation of functions and evolution of neuropeptides and hormones.

In this work, we present the identification of a novel VP/OT superfamily peptide, Ci-VP, and its endogenous receptor, Ci-VP-R, in *C. intestinalis*. To the best of our knowledge, this is the first report on the VP/OT superfamily of deuterostome invertebrates as well as protochordates.

2. Materials and methods

2.1. Animals

Adults of *C. intestinalis* were cultivated at the Maizuru Fisheries Research Station of Kyoto University and maintained in sea water at 18 °C.

2.2. Detection of Ci-VP by mass spectrometry

7 g of the Ciona neural complexes were pulverized by grinding under liquid nitrogen and extracted in 80 ml of methanol/water/acetic acid solution (90:9:1). The evaporated extract was separated by the Superdex^{TM} Peptide gel filtration column (GE Healthcare, Buckinghamshire, UK) and evaporated. The residue was dissolved with 0.1 M sodium bicarbonate (pH 9.0), and incubated for 30 min at 55 °C. 1/10 volume iodacetic acid was added to the peptide solutions, and were incubated for 15 min at room temperature. The solutions were directly applied to C18 reverse-phase column (Capillary Ex-Nano inertsil Peptides C18 0.2 mm × 150 mm; GL Sciences Inc, Torrance, CA, USA). The final peptides on an anchorchip were measured using the ultraflex III MALDI TOF/TOF machine (Bruker Daltonics, Bremen, Germany).

2.3. Functional analysis of Ci-VP-R expressed in oocytes of Xenopus laevis

The open reading frame region of Ci-VP-R cDNA was amplified and inserted into the Xenopus expression vector pSPUTK (Stratagene, La Jolla, CA, USA). The cRNA was prepared from the plasmid linearized with HpaI using SP6 RNA polymerase (Ambion, Austin, TX, USA). 50 nl of the cRNA solution (0.05 μg/μl) were injected into oocytes. The oocytes were incubated in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.6)) at 17 °C for 1–4 days. The oocytes expressed Ci-VP-R were voltage-clamped at −60 mV. The dose–response data and the EC_{50} values of the experiment were analyzed using Prism 3 software (GraphPad Software, San Diego, CA, USA) as previously reported [13,21].

2.4. Reverse-transcribed (RT)-PCR for the Ci-VP and Ci-VP-R mRNA

All PCR primers were ordered from SIGMA Genosis JAPAN (Tokyo, Japan). Total RNA extracted from the Ciona neural complex 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 (RACE) kit