The structure and expression of a preprohormone of a neuropeptide, Hym-176 in *Hydra magnipapillata*

Seungshic Yum, Toshio Takahashi, Masayuki Hatta, Toshitaka Fujisawa*

Department of Developmental Genetics, National Institute of Genetics, Mishima 411-8540, Japan

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Abstract Hym-176 (APFIFPGPKVamide) is a novel myoactive neuropeptide which was identified in systematic screening of signaling peptides in *Hydra magnipapillata*. By using PCR and library screening, we cloned and sequenced a full length cDNA which encoded a preprohormone of Hym-176. In the preprohormone, a typical signal sequence, one copy of Hym-176 precursor peptide and one copy of precursor sequence of another novel peptide, Hym-357 (KPAFLFKGYKPamide), were detected. In situ hybridization analysis revealed a strong signal in peduncle neurons. Signals were also detected, though weaker, in neurons in the gastric region and around the mouth. No signals were detected in the two extremities of the body, tentacles and basal disk. The expression pattern is correlated with the distribution of Hym-176 and its myoactive function.

Key words: Preprohormone; Neuropeptide; In situ hybridization; *Hydra*

1. Introduction

The phylum Cnidaria is the most primitive phylogenetic group which possesses a nervous system. It has been suggested that the cnidarian nervous system is predominantly peptidergic [1]. In fact, a large number of RFamides and other peptides ending with RXamide or KXamide have been isolated in Anthozoa and Hydrozoa [2]. RFamides are FMRFamide related peptides and are involved in myoactivity in anthozoans [3,4]. Recently, we have initiated a systematic screening of peptide signal molecules in a freshwater cnidarian, *Hydra magnipapillata*, by adopting a novel approach [5]. From the results obtained so far, we estimate that *Hydra* contains roughly 500 peptide signal molecules and that half of them are neuropeptides. Thus, we expect to identify many more neuropeptides in our screening program. Previously, we reported the isolation and characterization of the LWamide family [5,6]. The family consists of seven members of neuropeptides which share a common C-terminal motif of GLWamide. LWamides have two different functions: induction of metamorphosis of hydrozoan planula larvae into polyps and contraction of specific muscles in sea anemone and *Hydra* [5–8]. More recently, we reported another novel *Hydra* peptide named Hym-176 [9]. Hym-176 is a neuropeptide and has a primary sequence of APFIFPGPKVamide. It specifically and reversibly evokes contraction of ectodermal muscles of the peduncle region of *Hydra*.

Genes encoding RFamides and LWamides have been recently cloned and analyzed in various cnidarian species [10–14]. These genes are typical preprohormone genes in which a signal sequence and multiple copies of peptides are encoded. In the case of the LWamide gene in *Hydra* all seven members are encoded in a single preprohormone [14]. From the DNA sequence, one more putative LWamide is predicted but has not been isolated yet. On the other hand, there are three independent RFamide genes in *Hydra* [11]. Two of them are similar to each other, but the third one has a quite different repertoire of peptides except one that is common to all three genes.

In the present study, cDNA of the Hym-176 encoding gene was cloned, sequenced and its expression was examined with whole mount in situ hybridization. The details of these studies will be described.

2. Materials and methods

2.1. Animals and culture

Wild type strain 105 of *Hydra magnipapillata* was cultured as described previously [15]. This strain was used for all the experiments.

2.2. Strategy to clone cDNA encoding a peptide Hym-176

Since Hym-176 is a decapeptide and is too short for designing primers to amplify corresponding DNA, we extended the peptide by adding a possible processing sequence and an amidation sequence to the N-terminal and the C-terminal ends, respectively. The extended sequences have 16 amino acid residues as follows, where X can be any one of 20 amino acids: KK/D/RXAPFIFPGPKVGKK/R/D.

A degenerated oligonucleotide primer corresponding to the amino acids 2–7, 5′-(A/G)ANNN(A/T)GCNCCNTT(T/C)AT-3′, and a complementary primer corresponding to the amino acids 16–10, 5′-NTTC(T/C)TTNCCNAC(T/C)-3′, were designed. N stands for any one of four nucleotides.

Total RNA was extracted from polyps starved for 48 h by an AGPC method [16] and used as a template for first strand cDNA synthesis (First strand cDNA synthesis kit, Pharmacia). PCR was carried out using the first strand cDNA and *Taq* DNA polymerase (Boehringer-Mannheim) for 30 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 30 s. The amplified DNA was separated on a 1% agarose gel and the region slightly above the primers where no band could be seen was blindly cut out. DNA was purified using a Mermaid kit (BIO 101) and ligated to pCR2.1 (Invitrogen). After sequencing the clones, a sequence that corresponds to the entire Hym-176 peptide was obtained. Then, an oligonucleotide corresponding to amino acids 1–7 of Hym-176 (GCACCTTTCATTTTTCCTGG) was synthesized and used as a probe to screen a *Hydra* cDNA library (see blow).

2.3. Construction of cDNA library and screening

Total RNA was extracted as described above. Poly(A)$^+$ RNA was purified by using Oligotex (Roche). A cDNA library was constructed in Uni-ZAPII according to the manufacturer's instruction (Stratagene). The library was screened with the oligonucleotide probe labeled with [γ-^{32}P]dATP (NEN) using T4 polynucleotide kinase (Takara). Sequencing of positive clones was carried out with an ABI Prism 377 DNA sequencing system (Applied Biosystems).

2.4. Northern blot analysis

Poly(A)$^+$ RNA (3 μg) was fractionated on a formaldehyde-agarose

*Corresponding author. Fax: (81) (559) 81-6768.
E-mail: tfujisaw@lab.nig.ac.jp

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                    ACGAGCTCGTGCCGATTCGGCACGAGCGGCACGAGGAAAATCGAT   45
CTAGCTAGTTGAAAAACTCTCGACACATAATTATTACAAAGTAAAGAACGAAGTAAAAA  104
     *                                  *                *
ATG TCA AAA ATA AAT AAA CTA ACA ATG TAC GTG TTC TAT GCA TTA  149
 M   S   K   I   N   K   L   T   M   Y   V   F   Y   A   L    15
CTG GTT TTA AAC ATC TAC GTG GTT TTG TCT GTC AAC TCA TTG CCT  194
 L   V   L   N   I   Y   V   V   L   S   V   N   S   L   P    30
TTT CGC GAC GAT GAA GAT ACC GAT AAC GAA ATC GAT GGC GAC ATT  239
 F   R   D   D   E   D   T   D   N   E   I   D   G   D   I    45
AGC GAA TTA GAA AAT GAA TAT CAA ACC AAT CAA GTT TAT GAT TAT  284
 S   E   L   E   N   E   Y   Q   T   N   Q   V   Y   D   Y    60
AAC AAA TTT AAA AAC CAA GCA GAC TTG AAA ATC AAA GCT AGA AAT  329
 N   K   F   K   N   Q   A   D   L   K   I   K   A   R   N    75
CAC TAT GCT CCT TTC ATT TTT CCT GGG CCT AAA GTT GGT CGT GAT  374
 H   Y   A   P   F   I   F   P   G   P   K   V   G   R   D    90
GTT AAT TTT CAC TCA GTT TTA TCT CCA TCC GAC GAA TCA AGA AAA  419
 V   N   F   H   S   V   L   S   P   S   D   E   S   R   K   105
TCA TTC AAT AAT TAC CAT GAA AAC GGA TAC CGA CAT GAT AAA CCT  464
 S   F   N   N   Y   H   E   N   G   Y   R   H   D   K   P   120
GCA TTT TTA TTT AAA GGA TAT AAA CCT GGA GAT CAA ACA CAA AAG  509
 A   F   L   F   K   G   Y   K   P   G   D   Q   T   Q   K   135
AAT TTG TAA CTTCATTTAGTTTTTCAATTACCTTTATGCTATTTATTTAATGTAAT  565
 N   L   *                                                  137
CATGTGATATTTGTATAATTTTAAAGATTTATTAAGATAAGATTAGTTCTAACTAn     620
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Fig. 1. cDNA sequence of Hym-176 encoding gene and deduced amino acid sequence. The amino acid sequence of a Hym-176 precursor is underlined and printed in bold. The precursor sequence of another peptide, Hym-357, is also underlined. Stop codons are indicated by asterisks.

gel and blotted to Hybond N^+ nylon membrane (Amersham). A cDNA insert of a positive clone was labeled with [α-^{32}P]dCTP (Amersham) using a random primer labeling kit (Takara). Hybridization was carried out according to standard procedures [17]. The membrane was exposed overnight to an imaging plate and the image was analyzed by BAS-2000 (Fuji film).

2.5. In situ hybridization

Digoxigenin (DIG)-labeled antisense and sense riboprobes were prepared using a full length cDNA containing a Hym-176 precursor sequence according to the manufacturer's method (Boehringer-Mannheim). Whole mount in situ hybridization was performed by the method described by Grens et al. [18].

3. Results and discussion

As outlined in Section 2, cDNA encoding a neuropeptide Hym-176 was cloned. Fig. 1 shows the sequence of the sense strand of 619 bp excluding the poly(A)$^+$ stretch at the 3′ end. Since the first methionine codon starts from nucleotide number 106 and there are three stop codons in frame in its upstream 5′ region, this is very likely to be an initiation codon, although it is still possible that the second ATG (nucleotides 129–131) is an initiation codon. Assuming the first ATG to be an initiation codon, the cDNA contains an open reading frame of 138 amino acid residues long (Fig. 1). The nucleotide at position 338 of cDNA was different from that at position 3 of the oligonucleotide probe which had been used for screening the cDNA library. Mismatching of a primer appeared to occur during PCR. The deduced protein has a hydrophobic signal sequence at its N-terminus which is possibly cleaved off at S of amino acid position 25 or 28 [19]. One copy of the Hym-176 precursor sequence (underlined and printed in bold) was found in the protein. The sequence is flanked at the N-terminus by Y. N-terminal processing probably takes place at the C-terminal side of Y [2]. At the C-terminus, there is a possible amidation signal, GR, where the basic residue R probably serves as a substrate for processing enzymes and G serves as a donor of an amide group for amidation enzymes [2,20,21]. Near the C-terminus of the protein we also found a copy of another peptide, Hym-357 (underlined). Hym-357 had been isolated and its primary structure was determined to be KPAFLFKGYKPamide. At the C-terminus, the precursor sequence is flanked by GD. This strongly suggests that a new processing enzyme which removes an acidic amino acid instead of basic amino acid(s) is present in *Hydra* tissue. Detailed analyses of Hym-357, however, will be published elsewhere. Thus, the deduced protein is a typical preprohormone with a signal sequence and multiple copies of peptides. Whether or not other peptides are buried in the sequence remains to be seen. Database search revealed no known sequences homologous to the preprohormone, Hym-176 or Hym-357 sequences. This indicates that the gene and peptides are novel.

Northern blot analysis showed a single transcript of about 700 bp (Fig. 2). The result indicates that the sequence shown in Fig. 1 is a full length or nearly full length cDNA.

Expression of the Hym-176 coding gene in vivo was analyzed with whole mount in situ hybridization. Fig. 3b,c shows the results together with a schematic illustration of immunohistochemical staining of a polyp using anti-Hym-176 antibody (Fig. 3a) [9]. The overall distribution of nerve cells expressing the Hym-176 gene in the body column is shown in Fig. 3b. The following features are worth mentioning. (1) All the neurons expressing Hym-176 appear to be in the ectoderm. (2) No signal was detected in the tentacles. This is in

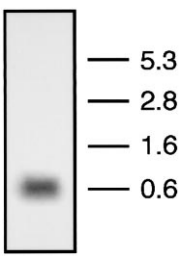

Fig. 2. Northern blot analysis of the Hym-176 transcript. The sizes of markers in kb are indicated at the right.

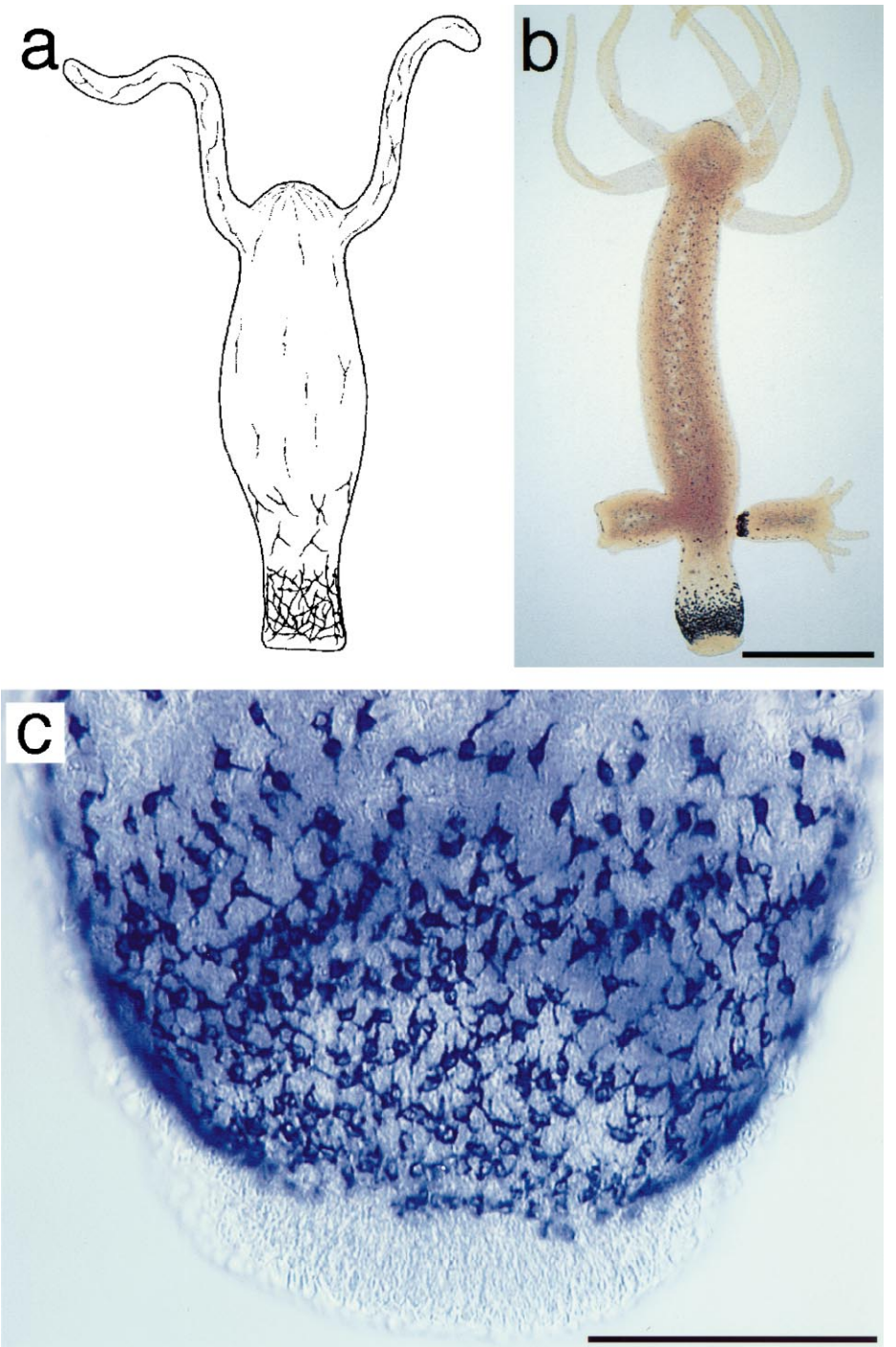

Fig. 3. Distribution and localization of Hym-176 peptides and transcripts in a *Hydra* body. a: Immunohistochemical staining using anti-Hym-176 antibody (an illustration adopted from Yum et al. [9]). b: Whole mount in situ hybridization. c: Whole mount in situ hybridization in higher magnification. Positive neurons in the lower peduncle region are shown. Bars in b and c respectively indicate 0.5 mm and 0.1 mm.

sharp contrast to immunostaining where some neurons were positive in the tentacles (Fig. 3a). This indicates that these neurons in the tentacles stably maintain the neuropeptide which was produced before the nerve cells were incorporated into tentacle epithelial cells at the base of tentacles [22]. Alternatively, the antibody may recognize other peptides which have a C-terminal sequence of PKVamide, a specific epitope for the antibody [9]. (3) A small number of ganglion cells around the mouth exhibited positive signals. (4) In the gastric region, signals were detected mainly in the cell bodies of ganglion cells. They are more or less evenly distributed and probably form a net-like structure. (5) Strong signals were seen in the neurons in the lower half of a peduncle. As a higher magnification shows (Fig. 3c), these cells have thick processes typical to this region [23,24]. Thus, mRNA was localized not only in cell bodies but also in some processes close to cell

bodies. (6) The size of cell bodies appears to become gradually smaller in more proximal regions. The reason for this size difference is not known. (7) The basal disk is free of positive neurons in both in situ hybridization and immunostaining. The basal disk, however, is not free of nerve cells because it contains GLWamide positive neurons [6]. Since epithelial cells together with nerve cells in the peduncle are displaced proximally into the basal disk, it is interesting to know the fate of Hym-176 positive neurons: whether they are phenotypically converted to Hym-176 negative cells or they cannot survive in the basal disk. Experiments to address these questions are under way. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB018544.

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