Three different prohormones yield a variety of Hydra-RFamide (Arg-Phe-NH$_2$) neuropeptides in _Hydra magnipapillata_

Dorothea DARMER*†, Frank HAUSER*, Hans-Peter NOTHACKER*†‡, Thomas C. G. BOSCH‡, Michael WILLIAMSON* and Cornelis J. P. GRIMMELIKKHUZEN*†‡

*Department of Cell Biology and Anatomy, Zoological Institute, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen Ø, Denmark, †Center for Molecular Neurobiology (ZMNH), University of Hamburg, Martinistrasse 52, D-20246 Hamburg, Federal Republic of Germany, and ‡Zoological Institute, University of Jena, Ernstbrunnenstrasse 1, D-07743 Jena, Federal Republic of Germany

The freshwater polyp _Hydra_ is the most frequently used model for the study of development in cnidarians. Recently, we isolated four novel Arg-Phe-NH$_2$ (RFamide) neuropeptides, the Hydra-RFamides I–IV, from _Hydra magnipapillata_. Here we describe the molecular cloning of three different prohormones from _H. magnipapillata_, each of which gives rise to a variety of RFamide neuropeptides. Preprohormone A contains one copy of unprocessed Hydra-RFamide I (QWLGGRFG), II (QWFNGRFG), III/IV [KP]HLRGRFG and two putative neuropeptide sequences (QLMSGRFG and QLMGRFG). Preprohormone B has the same general organization as preprohormone A, but instead of unprocessed Hydra-RFamide III/IV it contains a slightly different neuropeptide sequence [KP]HYRGRFG. Preprohormone C contains one copy of unprocessed Hydra-RFamide I and seven additional putative neuropeptide sequences (with the common N-terminal sequence QWF/LSGRFG). The two Hydra-RFamide II copies (in preprohormones A and B) are preceded by Thr residues, and the single Hydra-RFamide III/IV copy (in preprohormone A) is preceded by an Asn residue, confirming that cnidarians use unconventional processing signals to generate neuropeptides from their precursor proteins. Southern blot analyses suggest that preprohormones A and B are each coded for by a single gene, whereas one or possibly two closely related genes code for preprohormone C. Northern blot analyses and _in situ_ hybridizations show that the gene coding for preprohormone A is expressed in neurons of both the head and foot regions of _Hydra_, whereas the gene coding for preprohormones B and C are specifically expressed in neurons of different regions of the head. All of this shows that neuropeptide biosynthesis in the primitive metazoan _Hydra_ is already rather complex.

**INTRODUCTION**

Cnidarians are the lowest animal group to have a nervous system and it was probably within cnidarians, or a related ancestor phylum, that nervous systems first evolved [1]. The primitive nervous systems of cnidarians are strongly peptidergic. From a phylum, that nervous systems first evolved [1]. The primitive and it was probably within cnidarians, or a related ancestor

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Abbreviations used: RFamide, Arg-Phe-NH$_2$; SSC, 150 mM NaCl/15 mM sodium citrate (pH 7.0).

1 Present address: Institute for Pathophysiology, Martin Luther University of Halle-Wittenberg, Magdeburger Strasse 18, D-06112 Halle, Federal Republic of Germany.

2 Present address: Department of Pharmacology, College of Medicine, University of California, 354 Med. Surg. II, Irvine, CA 92697-4625, U.S.A.

3 To whom correspondence should be addressed at the University of Copenhagen.

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kinase (Stratagene) for 30 min at 37 °C. After being heated at 70 °C for 10 min, the unincorporated nucleotides were removed by passing the mixture through a NAP-5 column (Pharmacia).

**Screening of the Hydra cDNA library**

*H. magnipapillata* was cultured as described previously [10]. A λgt11 cDNA library from 1-day-starved *H. magnipapillata* was prepared as described earlier for sea anemones [11,12]. This library consisted of 2 × 10^6 primary plaques and was screened with a α^32P-labelled oligonucleotide, 5'-CCAGACCAACT-GGTAATG-3', corresponding to the presumed unprocessed amino acid sequence of Hydra-RFamide I, or with a labelled cDNA fragment. Plaque lifting and hybridization procedures were performed as described in [13]. Hybridization and final washing steps were at 39 °C for the oligonucleotide probe and at 60 °C for a cDNA fragment.

**Cloning of the 5' end of preprohormone B cDNA**

The 5' end of the preprohormone B cDNA was cloned by a nested PCR approach. The first round of amplification was performed with a λgt11 reverse primer, 5'-CCAGACCAACT-GGTAATG-3', and with the oligonucleotide corresponding to the immature Hydra-RFamide I sequence described above. The reaction mixture (20 μl) consisted of 1 × *Taq* buffer (Promega), containing 2.5 mM MgCl₂, 0.75 unit of *Taq* polymerase (Promega) and various concentrations of *Hydra* λgt11 cDNA library (10^4 or 2.5 × 10^5 plaque-forming units). The PCR parameters were: 30 cycles at 96 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The resulting PCR products were diluted 1:10 in *Taq* buffer and subjected to a second round of PCR with the same conditions as described above but replacing the primer corresponding to Hydra-RFamide I with an anti-sense primer, 5'-TGTTTCTAAGTAATCATTAC-3', corresponding to positions 387–366 in the preprohormone B cDNA sequence (see Figure 2) and containing three additional 5' nucleotides to give an EcoRI site for subcloning. The resulting PCR product of about 350 bp was digested with *Eco*RI, gel-purified, subcloned into pBluescript SK(+) (Stratagene) and sequenced.

**DNA sequencing**

cDNA insertions from positive λgt11 clones were excised with *Eco*RI and subcloned into pUC19 for sequencing. Subclones containing the largest cDNA insertion coding for preprohormone C were sequenced after the generation of *Exo*III/mung bean nuclease deletion clones. These clones were obtained after cutting with *Pst*I and *Ban*I and subsequent digestion with exonuclease III (Stratagene), in accordance with a protocol from the manufacturer (digestion was at 30 °C with six deletion time points of 90 s each). After this, blunt ends were produced with mung bean nuclease and re-ligation was performed with T4 DNA ligase (both from New England Biolabs). The deleted plasmids were subsequently transformed into *Escherichia coli* JM109 and prepared for sequence analyses. Sequencing was performed by the dideoxynucleotide chain termination method [14] with a T7 sequencing kit from Pharmacia and [α-^32P]dATP (Amersham). All subclones were sequenced in both directions. DNA sequence compilation, nucleotide and amino acid sequence comparisons, and database searches were performed with the DNASTAR program (DNASTAR).

**Northern blots**

Total RNA from *H. magnipapillata* and other species was prepared as described in [15]. Poly(A)^+ RNA was prepared by affinity chromatography with two passages through oligo(dT)-cellulose columns. The RNA samples and RNA marker III (Boehringer Mannheim) were denatured with glyoxal and DMSO and subjected to electrophoresis on 1.2% (w/v) agarose gels as described in [13]. The RNA was then blotted on a nylon membrane (Hybond N; Amersham) by capillary transfer and fixed to the membranes by UV irradiation as recommended by the manufacturer. Hybridization with labelled cDNA fragments was performed at 60 °C in a solution containing 6 × SSC (SSC is 150 mM NaCl/15 mM sodium citrate (pH 7.0)), 2 × *Denhardt’s* solution [Denhardt’s solution is 0.02% BSA/0.02% polyvinylpyrrolidone/0.02% Ficoll], 0.1% SDS, 0.01% herring sperm DNA and 50 mM sodium phosphate, pH 7.0. For hybridization, various α^32P-labelled cDNA fragments were used at a concentration of 5 × 10^6 c.p.m./ml. Washing was at 60 °C in 5 × SSC containing 0.1% SDS, with one final wash at 60 °C in 1 × SSC containing 0.1% SDS. Hybridization with oligonucleotide probes was performed at 42 °C in the same hybridization medium as for the cDNA fragments, except that 5 × *Denhardt’s* solution was used in place of 2 × *Denhardt’s*. With these probes, washing was at room temperature with 6 × SSC containing 0.1% SDS, with one final wash in the same washing solution at 40 °C. We used 5'-TGTTTCTAAGTAATCATTAC-3' as a specific oligonucleotide probe for preprohormone A, and 5'-AGGCCTAAATATCCTTTATATA-3' as a specific oligonucleotide probe for preprohormone B.

**Southern blots**

Genomic DNA from *H. magnipapillata* was prepared as described in [13], digested with various restriction enzymes and then separated on 0.8% agarose gels. Transfer to a Hybond-N membrane, hybridization with cDNA probes and washing were done as recommended by the supplier (Amersham). A final washing of the blots was performed with 0.1% SSC/0.1% SDS at 65 °C for 1 h.

**In situ hybridization**

Whole-mount *in situ* hybridization with digoxigenin-labelled RNA probes was performed as described in [16], with the following slight modifications. pBluescript vector (Stratagene) containing the required cDNA insertion was linearized by cleavage at a suitable position. Sense or anti-sense transcripts were obtained *in vitro* by using T3 or T7 RNA polymerase and the DIG RNA-labelling kit (Boehringer Mannheim). Hybridization was performed for 3 days at 55 °C in 50% formamide/5 × SSC/0.1% (v/v) Tween 20/0.1% CHAPS (Sigma)/1 × *Denhardt’s* solution/0.01% heparin/0.2 μg/ml labelled RNA probe. Staining was performed in the dark with BM-Purple (Boehringer Mannheim) as a substrate. Whole mounts were investigated and photographed with a Leitz DMRD/Leica DMRXA microscope.

**RESULTS**

Three different Hydra-RFamide preprohormones

We screened 1.3 × 10^6 phages of our *Hydra* λgt11 cDNA library with a degenerate oligonucleotide probe corresponding to the presumed immature sequence of Hydra-RFamide I (Gln-Trp-Leu-Gly-Gly-Arg-Phe-Gly). This yielded 28 positive clones, of which the cDNA insertions were subcloned and sequenced. Three different types of cDNA could be identified that each coded for a preprohormone containing one copy of immature Hydra-RFamide I and a variety of other neuropeptide sequences: 16 clones contained a cDNA coding for a preprohormone of type
Neuropeptide biosynthesis in Hydra

Figure 1 cDNA insertion of clone 23 from H. magnipapillata and deduced amino acid sequence for preprohormone A

The longest cDNA insertion coding for preprohormone A was 558 bp long. It was sequenced on both strands in one sequencing reaction each; its structure is shown in Figure 1. The cDNA contains an open reading frame of 492 bp and has an upstream untranslated sequence of 14 bp and an untranslated trailer of 52 bp. All other clones had untranslated regions that were shorter.

The open reading frame of the cDNA shown in Figure 1 codes for a preprohormone of type B and 8 for a preprohormone of type C. Below we describe the three precursor types.

Preprohormone A

The longest cDNA insertion coding for preprohormone A was 558 bp long. It was sequenced on both strands in one sequencing reaction each; its structure is shown in Figure 1. The cDNA contains an open reading frame of 492 bp and has an upstream untranslated sequence of 14 bp and an untranslated trailer of 52 bp. All other clones had untranslated regions that were shorter.

The open reading frame of the cDNA shown in Figure 1 codes for a preprohormone that is 164 amino acid residues long. The protein has an N-terminal hydrophobic signal sequence that is probably cleaved off at Ala or Ser.

The C-terminal region of the preprohormone contains the unprocessed sequences of Hydra-RFamide I (amino acid positions 114–121 of Figure 1), Hydra-RFamide II (amino acid positions 127–134) and Hydra-RFamides III and IV (amino acid positions 151–159 and 153–159). All these neuropeptide sequences are followed by an Arg residue, which is an established cleavage site, but are preceded by Asp (Hydra-RFamide I), Thr (Hydra-RFamide II), or Asn (Hydra-RFamide III–IV) residues, which must therefore represent novel cleavage sites.

The preprohormone contains, in addition to the Hydra-RFamide I–IV sequences (printed in bold type and underlined in Figure 1), two putative neuropeptide sequences (Figure 1, underlined only). These putative neuropeptide sequences are followed by one or two basic amino acid residues, suggesting that they will be released from the precursor and converted into mature neuropeptides (see also the Discussion section).

Preprohormone B

This cDNA is composed of the cDNA insertion of Agt11 cDNA library clone 32 (nt 137–628) and of a 5′ PCR clone (nt 1–365). Hydra-RFamide I and II sequences are underlined and printed in bold. Highly likely, but putative, neuropeptide sequences are underlined only. Another possible putative neuropeptide sequence is underlined by a broken line. This sequence is less certain because it is preceded by a Ser residue. Stop codons are indicated by a dot.

Preprohormone C

A, 4 coded for a preprohormone of type B and 8 for a preprohormone of type C. Below we describe the three precursor types.

Figure 2 Composite cDNA coding for preprohormone B

All four cDNA insertions coding for preprohormone B failed to contain the start codon of the open reading frame. A second round of screening of 10′ phages with a cDNA insertion coding for preprohormone B (nt 196–615 of Figure 2) yielded three
additional clones with insertions coding for preprohormone B, which again, however, did not contain the start codon. Therefore we used an anchored PCR approach, which finally yielded the 5’ end of the precursor protein cDNA. Figure 2 shows the composite cDNA coding for the whole preprohormone. The cDNA is 628 bp long and contains a non-coding 5’ sequence of 123 bp and a non-coding 3’ sequence of 7 bp. The non-coding 5’ sequence contains two stop codons, of which one is located shortly before the start codon (Figure 2).

The open reading frame of the cDNA codes for a protein of 166 amino acid residues. This protein has a signal sequence that is probably cleaved off at Ser169 [17]. Preprohormone B has an organization very similar to that of preprohormone A: the C-terminal region of the protein contains unprocessed Hydra-RFamide I (at amino acid positions 116–123 of Figure 2) and Hydra-RFamide II (at amino acid positions 129–136). These two sequences are followed by basic amino acid residues and preceded by Asn or Thr residues that, as we have seen in preprohormone A, can be cleavage sites.

There is an amino acid sequence (at amino acid positions 373–380; printed in bold type and underlined in Figure 3) that contains a stop codon. This sequence is followed by a basic amino acid residue (Arg) and preceded by an acidic residue (Glu), showing that it can be released from the precursor protein. In addition to the single copy of Hydra-RFamide I, the precursor contains seven putative neuropeptide sequences that are very regularly distributed over the central part of the precursor protein (at amino acid positions 100–111, 139–150, 178–189, 217–228, 256–267, 295–306 and...
334–345 in Figure 3). These sequences are followed by pairs of basic residues (Lys-Arg) and preceded by acidic residues (Glu), suggesting that they will be released from the precursor.

### Northern blot analyses

mRNA was isolated from whole *H. magnipapillata* and analysed in a Northern blot. Both a cDNA probe coding for preprohormone A (nt 24–537 of Figure 1) and a probe coding for preprohormone B (nt 196–615 of Figure 2) hybridized with mRNA of approx. 800 bases (Figure 4). The cDNA coding for preprohormone C (nt 1–1303 of Figure 3) hybridized with a mRNA population of approx. 1600 bases (Figure 4). These findings show that we have cloned almost the full lengths of the cDNA species coding for the three preprohormones, if one assumes that the three mRNA species coding for the three precursors have a poly(A)$^+$ tail of approx. 250 bases, which is normally true in eukaryotes.

The three types of cDNA were also used as probes in Northern blots containing, in addition to mRNA from *H. magnipapillata* (*Hydrozoa, Cnidaria*), whole-animal mRNA species from the hydromedusa *Polyorchis penicillatus* (*Hydrozoa, Cnidaria*), the scyphomedusa *Cyanea lamarckii* (*Scyphozoa, Cnidaria*), the sea anemones *Anthopleura elegantissima* and *Calliactis parasitica* (*Anthozoa, Cnidaria*), and mRNA from rat brain. Only mRNA from *H. magnipapillata* gave hybridization signals (Figure 4).

*Hydra* consists of a tube-like body column (gastric region) that is connected at one end to a head (with mouth and tentacles) and at the other end to a foot. We cut *Hydra* into three portions of equal length containing head, foot or gastric regions, isolated RNAs from these fragments and analysed them in Northern blots (Figure 5). The complete cDNA species coding for preprohormones A and B show 80% nucleotide identity and might cross-react. For the experiments of Figure 5 we therefore used anti-sense oligonucleotide probes that were specific for mRNA coding for either preprohormone A (a probe corresponding to nt 131–152 of Figure 1) or preprohormone B (a probe corresponding to nt 240–261 of Figure 2). We used the whole cDNA insertion of Figure 3 as a probe specific for preprohormone C mRNA. The specificities of these probes were confirmed by using dot-blot analyses. The specific oligonucleotide probe for preprohormone A hybridized with head and foot mRNA species but not with mRNA from the gastric region. Both the oligonucleotide probe specific for preprohormone B and the cDNA probe specific for preprohormone C hybridized with mRNA from the head, but not with mRNA species from the foot or gastric regions (Figure 5). The mRNA coding for preprohormone B seemed to be less abundant in *Hydra* than the mRNA species coding for preprohormones A and C.

### Whole-mount in situ hybridization

We obtained no good *in situ* hybridization signals in whole-mounted *Hydra* by using the single-stranded anti-sense oligonucleotide DNA probes specific for either preprohormone A mRNA or preprohormone B mRNA (see Northern blots, Figure 5). We therefore used anti-sense RNA probes produced by the transcription *in vitro* of cDNA insertions coding for each of the three preprohormones. This gave excellent *in situ* hybridization signals but the expected disadvantage was that the probes coding for preprohormones A and B cross-reacted slightly with the same mRNA species (again, the probe coding for preprohormone C was specific). By using an RNA probe directed against the mRNA of preprohormone A (corresponding to nt 2–537 of Figure 1), we found strong hybridization signals in neurons located in the upper gastric region (just below the tentacles and...
Figure 6  In situ Hybridization of whole-mount H. magnipapillata with anti-sense RNA probes directed against the mRNA species of preprohormones A (corresponding to nt 2–537 of Figure 1), B (corresponding to nt 146–614 of Figure 2) or C (corresponding to nt 1–1303 of Figure 3)

(A) Overview of a whole Hydra bearing a young bud hybridized with a probe directed against preprohormone A mRNA. Hybridization occurs in neurons of the basal two-thirds portion of the tentacles, in the hypostome, in neurons of the upper part of the gastric region just below the tentacles and in the foot (in an area called the peduncle, just above the basal disk). Staining is absent from most of the gastric region. In the young bud the same staining pattern is observed. Magnification ×45. (B) Overview of a whole Hydra bearing a young bud, hybridized with a probe directed against preprohormone B mRNA. Hybridization can be seen in the hypostome and in neurons of the upper gastric region just below the tentacles. There is a very weak hybridization signal in the peduncle and no hybridization in the tentacles and most of the gastric region. A similar staining pattern is observed in the young bud: note that staining is absent from foot and tentacles. Magnification ×45. (C) Overview of a whole Hydra hybridized with a probe directed against preprohormone C mRNA. There is staining exclusively in neurons of the tentacles. Note that the hybridization signals seem to be stronger in the tips of the tentacles, where clusters of strongly hybridizing neurons occur. Note also the clear border between tentacles and hypostome. Staining is completely absent from the hypostome, the whole gastric region and the foot. Magnification ×45. (D) A very early bud, before the emergence of its tentacles, hybridized with a probe directed against preprohormone A. Note that a region at the tip of the bud is stained in a ring-like fashion. At its borders, four regularly distributed neurons can be seen. In the right corner is the strongly hybridizing foot of the adult Hydra. Magnification ×110. (E) Detail of the upper gastric region just below the tentacles hybridized with a probe directed against preprohormone B mRNA. Strong hybridization signals can be seen in neurons, whereas hybridization is absent from the tentacles. Magnification ×110. (F) A young bud hybridized with a probe directed against preprohormone C mRNA. Note the very prominent staining of neurons in the early tentacles and the absence of staining from the rest of the body. Magnification ×100.

mouth), in the mouth area (hypostome), in neurons of the basal two-thirds portion of the tentacles and in neurons of the foot (in an area called the peduncle, just above the basal disk) (Figures 6A and 6D). Hybridization was absent from the tentacle tips of adult animals, the mid-gastric region and basal disk. The staining of the hypostome area could be seen already in very early buds, where it had a ring-like or doughnut-like shape (Figure 6D).

Using an RNA probe directed against preprohormone B mRNA (corresponding to nt 146–614 of Figure 2), we found strong staining in the hypostome and upper gastric region (just below the head) but little or virtually no staining in the tentacles and foot (Figures 6B and 6E). This suggested that the slight staining in the tentacles and foot was due to cross-reaction with preprohormone A mRNA and that the preprohormone B gene was expressed only in neurons of the mouth area (hypostome) and upper gastric region, which is in agreement with the data from our Northern blots (Figure 5B). By using an RNA probe specific for preprohormone C mRNA (corresponding to nt 1–1303 of Figure 3), we found that staining exclusively occurred in neurons of the tentacles. This staining was especially strong in the apical parts...
of the tentacles, with a cluster of positive neurons frequently being located in the tentacle tip (Figure 6C). The production of preprohormone C mRNA occurred very early in tentacle development, as could be seen after staining of early developing buds (Figure 6F). No staining was observed when we used sense instead of anti-sense RNA probes corresponding to preprohormone A, B or C mRNA. The distribution of mRNA species coding for preprohormones A, B and C (Figure 6) corresponds well to the distribution of neurons stained with an antibody against RFamide peptides [7].

Southern blot analyses

The cDNA probe coding for preprohormone A (nt 2–537 of Figure 1) hybridized with single DNA fragments obtained after digestion of genomic Hydra DNA with EcoRI, BamHI, HindIII, XhoI, SacI (Figure 7A) or PstI (results not shown), suggesting the presence of one single gene coding for preprohormone A.

When a cDNA fragment coding for preprohormone B (nt 146–614 of Figure 2) was used as a probe in our Southern blot analysis, single hybridizing bands were obtained after digestion of genomic DNA with EcoRI, BamHI, XhoI, SacI (Figure 7B) or PstI (results not shown) that had about the same size as those hybridizing with the cDNA probe coding for preprohormone A (Figure 7A). However, four hybridizing bands appeared in genomic DNA cleaved by HindIII. One of them was a weakly hybridizing band (band 1 in Figure 7B) with the same size as the strongly hybridizing band (band 1) in Figure 7A. Its presence could be explained by cross-reaction of the preprohormone B probe with the HindIII fragment of the DNA coding for preprohormone A (band 1 in Figure 7A). The other three bands are strongly hybridizing (bands 2–4 in Figure 7B) and their presence could be explained by two HindIII restriction sites in the gene coding for preprohormone B: one located inside the region corresponding to the DNA shown in Figure 2 (there is a HindIII restriction site at nt 299–305 of Figure 2) and another located within a presumed intron between positions 146 and 614 of the DNA shown in Figure 2. The hybridizing bands 1–4 in Figure 7B could therefore be explained on the basis of one single gene coding for preprohormone B.

When the cDNA coding for preprohormone C (nt 1–1303 of Figure 3) was used as a probe, single hybridizing bands appeared in a Southern blot using genomic DNA digested with XhoI, SacI (Figure 7C) and PstI (results not shown). However, when genomic DNA digested with EcoRI, BamHI or HindIII was used, two (with EcoRI or BamHI) or four (with HindIII) hybridizing bands appeared (Figure 7C). The presence of two or four hybridizing bands can be explained in two ways. First, there could be one gene coding for preprohormone C that, in the region corresponding to the cDNA of Figure 3, contains at least three introns. Each of these three introns has a HindIII restriction site giving rise to four hybridizing fragments, whereas only one of the introns has an EcoRI and/or BamHI restriction site, giving rise to two hybridizing fragments. The other possibility is that there are two or more closely related genes hybridizing with the preprohormone C probe.

DISCUSSION

Novel prohormone-processing sites

From earlier work on the biosynthesis of cnidarian neuropeptides, we have seen that cnidarians use a variety of unconventional cleavage signals for the generation of their neuropeptides. These novel cleavage sites are Asp and Glu residues [11,12,19,20], Asn and Ser residues [10] and N-terminal Xaa-Pro or Xaa-Ala sequences [21]. These unconventional cleavage signals are always located at the N-terminal sides of the unprocessed neuropeptide sequences, whereas at the C-terminal flanking regions conventional monobasic or dibasic processing sites occur. The enzyme responsible for the removal of N-terminal Xaa-Ala or Xaa-Pro sequences is most probably a dipeptidyl aminopeptidase [21], whereas the other enzymes are hitherto unknown aminopeptidases or endoproteinases [2].

Because we have recently isolated and sequenced four Hydra-RFamide peptides from H. magnipapillata [6], we are now able to draw conclusions on the maturation of their precursors. Each single copy of immature Hydra-RFamide I located on preprohormones A, B and C is followed by a monobasic processing site and preceded by Asp, Glu or Asn (Table 1), confirming our earlier findings that processing at these residues is possible in cnidarian prohormones. Each single copy of immature Hydra-RFamide II located on preprohormones A and B is followed by a monobasic processing site but is preceded by a Thr residue (Table 1). This means that Thr residues also must function as a cleavage signal. Cleavage at Thr residues, however, is not surprising because Thr closely resembles Ser, which, as mentioned above, has already been recognized as a cleavage site for cnidarian prohormone processing [10]. The single copy of immature Hydra-RFamide III in preprohormone A is followed by a single basic processing site and preceded by an Asn residue (Table 1), in agreement with our earlier conclusions that Asn can be a cleavage signal in cnidarian precursor proteins [10]. The single copy of immature Hydra-RFamide IV (HLRGRFG) on preprohormone A can be generated from Hydra-RFamide III (KPHLRGRFG) by the removal of an N-terminally located Lys-Pro sequence which, as
proposed earlier [21], can be catalysed by a dipeptidyl aminopeptidase.

It is very possible that the enzymes responsible for the above-
mentioned cleavages at Asp/Glu, Thr and Asn residues are aminopeptidases that exert their actions after the elongated Hydra-RFamide I–III sequences have been excised at their flanking basic amino acid residues (Table 1 and Figures 1–3) by conventional endoproteolytic cleavage [18]. However, the structure of another cnidarian preprohormone, the sea anemone prepro-Antho-RPamide I (where there is only one copy of the established, unprocessed Antho-RPamide I sequence, which is preceded by acidic amino acid residues without the presence of basic residues), suggests that cleavage at Asp/Glu residues is catalysed by an endoprotease [2]. The removal of the N-terminal extensions of the immature Hydra-RFamides I–IV sequences might therefore occur by the concerted actions of both endo- and exo-peptidases.

**Novel neuropeptide sequences**

In addition to the Hydra-RFamide I–IV sequences in prepro-
hormone A, there are two putative neuropeptide sequences (at amino acid positions 51–58 and 96–103 of Figure 1) that are followed by basic processing sites and preceded by either a Glu or an Arg residue. Thus it is very likely that these sequences also are released from the precursor protein. Their mature structures are probably < Glu-Leu-Leu-Met-Ser-Gly-Arg-Phe-NH$_2$ (Hydra-RFamide VII; Tables 1 and 2). Finally, there is an amino acid sequence that closely resembles the Hydra-RFamide III/IV sequence of preprohormone A (at amino acid positions 153–161 of Figure 2). This sequence has the same processing signals as the immature Hydra-RFamide III/IV sequence and is therefore likely to be released and converted into Lys-Pro-His-Tyr-Arg-Gly-Arg-Phe-NH$_2$ (Hydra-RFamide VIII) and His-Tyr-Arg-Gly-Arg-Phe-NH$_2$ (Hydra-RFamide IX; Tables 1 and 2).

Preprohormone C is remarkable because it contains, in addition to the single copy of Hydra-RFamide I, seven totally novel neuropeptide sequences (Figure 3). Each of these putative neuropeptide copies is followed by a dibasic processing site and preceded by a Glu residue (Table 1), which makes likely that they are released from the precursor protein. The N-terminal regions of the seven peptides show a striking similarity to members of the Hydra-RFamide family, especially Hydra-RFamides I and II (Table 2). The mature neuropeptides, however, probably do not carry an amidated C terminus, because a C-terminal Gly residue, which is necessary for amidation [22,23] is lacking at the appropriate positions (positions 111, 150, 189, 228, 267, 306 and 345 of Figure 3) in the unprocessed sequences (see also Table 1). Instead, the mature neuropeptides are likely to be C-terminally elongated compared with the Hydra-RFamides (Tables 1 and 2). It is difficult to give a prediction of the C-terminal regions of these seven putative neuropeptides. The last amino acid residues might be Glu or His; however, if processing occurs at Asn or Thr residues the C-terminal regions might be shorter. Because the biological action of most amidated neuropeptides is dependent on their C-terminal amidation, one can expect that the putative C-terminally elongated, non-amidated Hydra peptides have an action different from that of the Hydra-RFamides.

Table 1 N- and C-terminal extensions of Hydra-RFamides I–IV and related putative neuropeptide sequences

<table>
<thead>
<tr>
<th>Name</th>
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<tr>
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<tr>
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The immature Hydra-RFamide I sequence of preprohormone A was flanked by similar processing sites. Furthermore there is a neuropeptide sequence (at amino acid positions 96–103 of Figure 2) that is nearly identical with the Hydra-RFamide VI sequence of preprohormone A. It is flanked at both sides by basic amino acid residues and its mature structure is probably < Glu-Leu-Leu-Arg-Gly-Arg-Phe-NH$_2$ (Hydra-RFamide VII; Tables 1 and 2). Finally, there is an amino acid sequence that closely resembles the Hydra-RFamide III/IV sequence of preprohormone A (at amino acid positions 153–161 of Figure 2). This sequence has the same processing signals as the immature Hydra-RFamide III/IV sequence and is therefore likely to be released and converted into Lys-Pro-His-Tyr-Arg-Gly-Arg-Phe-NH$_2$ (Hydra-RFamide VIII) and His-Tyr-Arg-Gly-Arg-Phe-NH$_2$ (Hydra-RFamide IX; Tables 1 and 2).
sequences, of which three have the sequence KRNEVNPMEIKKDSDTERRFNRESLE (at amino acid positions 190–216, 229–255 and 307–333 of Figure 3), whereas the others have very similar sequences (at amino acid positions 73–99, 112–138, 151–177, 268–294 and 346–372). Database searches (SwissProt) did not reveal any significant similarity of any of these sequences with known proteins or peptides. It could be that these sequences represent novel, biologically active peptides. However, because these sequences are not protected by a < Glu group and also contain many of the processing sites discussed earlier (Lys, Arg, Asn, Glu, Asp, Ser and Thr), it could be that they merely have a spacing function and that they are degraded during processing of the prohormone.

Comparison between the three preprohormones

It is clear that the overall organizations of the preprohormones A and B are much more similar to each other than to that of preprohormone C. This is confirmed by sequence alignments, where we found 70% amino acid residue identity between preprohormones A and B and only 20–56% identity when we compared whole preprohormone A (or B) with different portions of preprohormone C. The same conclusion was reached when we aligned the cDNA sequences of the three preprohormones. All of this suggests that the genes coding for preprohormones A and B have originated from the same gene duplication event, whereas the gene coding for preprohormone C has been involved in another (perhaps earlier) gene duplication.

Preprohormone gene expression

Our Southern blot analyses (Figure 7) suggest that preprohormones A and B are each coded for by a single gene, whereas preprohormone C might be coded for by one or possibly two or more closely related genes. The three (or more) preprohormone genes are differentially expressed. The preprohormone A gene is expressed in neurons of both the head and foot regions, whereas the preprohormone B and C genes are specifically expressed in neurons of the head (Figures 5 and 6). This means that the putative, elongated Hydra peptides (Hydra-RF-elongated I and II), but also Hydra-RFamides V, VII, VIII and IX (Table 2) are produced only in the head region. Head-specific or foot-specific neurohormones are interesting because they are good candidates for factors that control morphogenesis and cell differentiation in Hydra [4,5,24]. Recently it has been found that a novel group of Hydra neuropeptides with the C-terminal sequence Leu-Trp-NH₂ (LWamide) influences gene expression in Hydra [25]; in the marine hydroid Hydractina, LWamide neuropeptides induce metamorphosis in planula larvae to become primary polyps [26,27]. These findings show that it is very possible that cnidarian neuropeptides have a role in development. Our work on the cloning of the three Hydra-RFamide preprohormones could therefore be the basis for future studies on the involvement of these proteins and their derived neuropeptides in Hydra development.

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**REFERENCES**


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**Table 2** Established and putative neuropeptides that could be released from prohormones A, B and C

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D. Darmer and others

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