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Molecular cloning of crustacean putative molt-inhibiting hormone (MIH) precursor

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A cDNA encoding the complete precursor of the putative molt-inhibiting hormone (MIH) of the shore crab, *Carcinus maenas*, was isolated and sequenced. The precursor consists of a putative 35 amino acid signal peptide and the 78 amino acid mature MIH. The deduced MIH amino acid sequence is in complete agreement with the sequence previously determined by Edman degradation. *In situ* hybridization revealed MIH-expression in a subpopulation of large neurosecretory perikarya of the medulla terminalis X-organ in the eyestalk.

Molt-inhibiting hormone; Neuropeptide precursor; cDNA sequence; Crustacea

1. INTRODUCTION

The current consensus model regarding the control of molting in decapod crustaceans comprises the following tenets. The control is basically negative, i.e. there is a molt-inhibiting hormone (MIH) which keeps the animal in intermolt. It originates in the eyestalk ganglia, where it is produced by neurosecretory cells in the so-called medulla terminalis X-organ (MTXO). MIH is secreted from axon terminals in the sinus gland (SG), a neurohemal organ in the eyestalk. Inhibition of molting is thought to be due to a suppressive action of circulating MIH on ecdysteroid synthesis in the molting glands (Y-organs). The mechanism of this inhibitory action is not yet understood in detail (see [1–3] for reviews).

Attempts to isolate and characterize MIH have consequently been based on bioassays in which the suppression of ecdysteroid synthesis was measured. For the shore crab, *Carcinus maenas*, an efficient in vitro bioassay, using isolated Y-organs, was developed [4] and proved useful for the isolation of a peptide with potent ecdysteroid synthesis-inhibiting activity from SG of this species [5]. The amino acid sequence of this putative MIH has recently been reported [6]. It is designated putative because its expected physiological in vivo role in the regulation of the natural molting cycle remains to be demonstrated. The sequence of *Carcinus* MIH revealed similarity to a longer known peptide from the same species, the crustacean hyperglycemic hormone (CHH) [7] and to other MTXO peptides from different species. Together, they constitute a novel peptide family (CHH/MIH/VIH; see [8] for review). *Carcinus* MIH and CHH display a sequence homology of 28% and a limited overlapping biological activity insofar as CHH has some MIH activity [5].

In order to study the genetic basis of both the intra- and interspecific relationship of the MTXO peptides, and thus their structural and functional divergence, investigations on precursor structures by cDNA cloning are desirable. We have previously elucidated the complete structure of the *Carcinus* CHH precursor [9] and describe here the complete MIH precursor of this species by isolation of a full length clone from the same cDNA-library.

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Abbreviations: CHH, crustacean hyperglycemic hormone; CPRP, CHH precursor related peptide; GIH (=VIH), gonad-inhibiting hormone (vitellogenesis-inhibiting hormone); MIH, molt-inhibiting hormone; MTXO, medulla terminalis X-organ; PCR, polymerase chain reaction; SG, sinus gland; SP, signal peptide.

2. MATERIALS AND METHODS

2.1. *Amplification of cDNA by the polymerase chain reaction (PCR)*

To amplify cDNA encoding MIH, DNA of a previously generated library from MTXO neurosecretory cells of *Carcinus maenas* [9] served as template for the PCR. Two degenerated oligonucleotides, deduced from the amino acid sequence of *Carcinus* MIH [6] were used as primers (M1: 5′-GGGAATTC(C/A)G(A/C/G/T)GT(A/C/G/T)AT(T/C/A)AA(T/C)GA(T/C)GA(A/G)TG(T/C)CC-3′, sense, encoding amino acids 1–8 and M2: 5′-CCGGATCC(A/C/G/T)GC(A/C/G/T)CC(A/C/G/T)A(A/G)(A/G/T)AT(A/C/G/T)CC(A/C/G/T)ACCC-3′,antisense, encoding amino acids 69–76 of MIH). The reaction was carried out for 30 cycles with a denaturation step of 40 s at 93°C, an annealing step of 2 min at 60°C, and an extension step of 3 min at 72°C with a final extension of 7 min. PCR products were subcloned into pBluescript KS+ and sequenced.

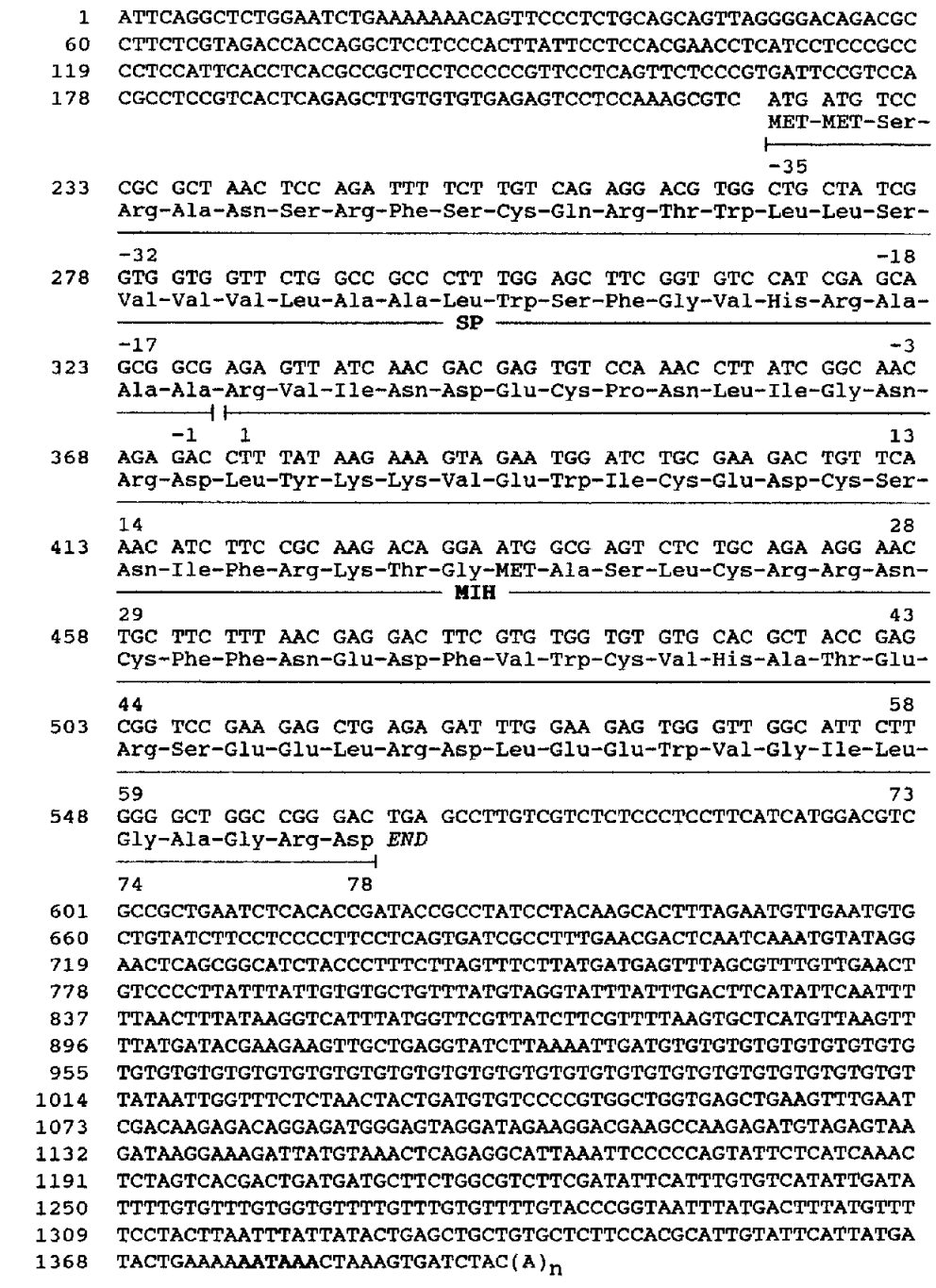

Fig. 1. Nucleotide- and deduced amino acid-sequence of the cDNA encoding the MIH precursor of *Carcinus maenas*. The sequences of the putative signal peptide (SP) and the MIH are underlined. The putative polyadenylation site is marked by **bold-face type**.

2.2. *PCR-based cDNA-library screening*

Based on the sequence data obtained from one clone (FRC7NP), a specific oligonucleotide (M3: 5′-TCCTTCTGCAGAGACTCGC-3′, antisense, corresponding to bp 437 455 in Fig. 1) was designed and used in combination with primer M1 for a PCR-based screening of the cDNA-library. DNA of the MTXO-cDNA-library, divided into 20 batches, each containing 5000 independent clones, was used as template under the same conditions as described above. Batches which yielded an amplified product of the predicted length were subjected to cDNA-library screening by colony hybridization.

2.3. *Screening by colony hybridization*

Clones of two batches, selected according to the results of the PCR-based cDNA-library screening, were screened by colony hybridization with a ^{32}P-labelled probe [11]. The probe was constructed by labelling the insert of clone FRC7NP with [^{32}P]dATP by use of the

random priming technique [12,13]. Plasmid-DNA of positive clones was subcloned in pBluescript KS+ and sequenced on both strands. All sequences were determined by the dideoxy sequencing method [14].

2.4. *In situ hybridization*

A RNA-probe was synthesized as run-off transcript from linearized recombinant pBluescript II KS+ vectors including the cDNA sequence between primers M1 and M2 and labelled with digoxigenin-11-dUTP [15]. Optic ganglia from eyestalks of *Carcinus maenas* in intermolt stage (C4) were dissected and immediately fixed in Bouin's fixative for 16 h. After dehydration in ethanol series, fixed eyestalks were embedded in paraffin and 5-μm sections were collected on slides coated with 0.01% poly-L-lysine and baked overnight at 45–50°C. Mounted sections were deparaffinized and rehydrated. For hybridization of the specific MIH-mRNA the sections were pretreated with pepsin, rinsed in phosphate-buffered saline (PBS), postfixed, treated with hydroxylammonium chloride, finally rinsed in PBS, and dehydrated. Hybridization was performed at 50°C for 16 h using digoxigen labelled cRNA probes at a concentration of 2 ng/μl hybridization buffer. After washing of the slides and treatment with 50 μg/ml RNase A for 30 min at 37°C, visualization of the probe was performed as described by Tensen et al. [16].

3. RESULTS

The clone whose sequence is shown in Fig. 1 was isolated from a cDNA library derived from neurosecretory MTXO cells of *Carcinus maenas* by a combination of PCR-based screening and colony hybridization. It was sequenced in both directions, using specific oligonucleotides as primers. This clone consists of a total of 1396 bp (excluding the poly(A) tail) and an open reading frame of 339 nucleotides. The open reading frame comprises the complete MIH precursor sequence, consisting of a signal peptide of 35 amino acid residues and the 78 residue mature MIH. A 5′-flanking sequence of 223 bp is present, as well as a 3′-untranslated region of 831 bp following the stop codon.

Expression of the MIH gene was visualized in MTXO-perikarya in the eyestalk of *Carcinus maenas* (Fig. 2) by in situ hybridization with a digoxygenin labelled antisense cRNA probe, corresponding to bp 329–555 of the cDNA clone (Fig. 1).

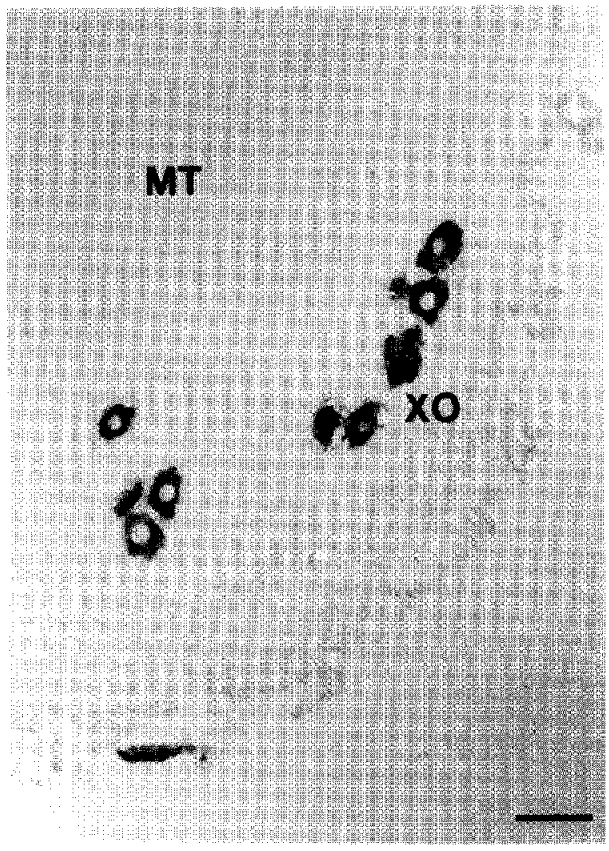

Fig. 2. Visualization of MIH-gene expressing perikarya in the eyestalk ganglia of *Carcinus maenas* by in situ hybridization. MT, medulla terminalis neuropil; XO, X-organ; bar = 50 μm

4. DISCUSSION

The MIH precursor of *Carcinus maenas* is a relatively simple one, consisting of a signal peptide which is directly followed by the MIH sequence. The sequence of the hormone, as deduced here from the cDNA, confirms the sequence obtained previously by Edman degradation of the mature peptide which was isolated from sinus glands [6].

Since MIH has been recognized as a member of a peptide family that also includes crustacean hyperglycemic hormones (CHH) and vitellogenesis-inhibiting hormone (VIH) (see [8] for review), the elucidation of the prohormone structure could be expected to yield more insights into the genetic relationship of these peptides. In *Carcinus*, two members of the family are known, i.e. MIH [6] and CHH [7]. The latter is a 72 amino acid peptide while MIH posesses 78 residues. A further difference is the presence of both N-terminal and C-terminal blocking groups in CHH which are lacking in MIH. Both peptides share 28% of identical amino acid residues.

The cDNA structure of the CHH precursor has previously been established in our laboratory [9]. Comparison of CHH and MIH precursors reveals striking and interesting differences. In the CHH precursor, a 38-residue peptide and a dibasic processing site is encoded between the signal peptide and the CHH sequence. For this peptide, referred to as CHH precursor related peptide (CPRP), no biological function has been found yet. Surprisingly, a sequence similar or homologous to CPRP is completely lacking in the MIH precursor.

The signal sequence of the MIH precursor is longer compared to that of the CHH precursor (35 vs. 26 amino acids). Another difference concerns the region preceding the stop codon: in the CHH precursor there is a Gly (as C-terminal amide donor) followed by Arg-Lys-Lys and the stop codon, whereas in the MIH precursor the codon for the last amino acid, Asp (which remains unamidated), is directly followed by the stop

codon. This difference may reflect the lack of an amidation mechanism in the processing of the MIH precursor.

In conclusion, comparison of the MIH and CHH precursor structures reveals a lower degree of genetic relationship than could have been expected from the comparison of the mature hormones.

Clearly, more precursors of CHH/MIH/VIH peptides have to be elucidated before generalizations are possible. A few further comments, however, appear to be warranted on the basis of existing data. Comparison of known structures has revealed that *Carcinus* MIH is much more similar to VIH of *Homarus* than to CHH of *Carcinus* or to any of the other CHHs which together form a highly homologous group; i.e. an MIH/VIH type of peptide appears to be clearly distinct from a CHH type [8]. The result of the present study suggests that the distinction appears to be even more pronounced at the precursor level. To confirm this, it would be highly interesting to know the structure of the VIH precursor. Although CHH precursors other than that of *Carcinus* [9] have not yet been published, there is indirect evidence that they share structural characteristics, in particular the presence of CPRPs. Such peptides have been isolated and sequenced from the sinus glands of four species [17]. They are homologous, and their stochiometric relationship to the CHH peptides indicate that they are part of the precursors.

In situ hybridization reveals MIH-expression in a subpopulation of the large neurosecretory MTXO perikarya (Fig. 2). No other MIH-expressing cells were observed in the entire eyestalk. These results agree with previous studies using peptide-immunocytochemistry [18]. In a separate paper we have studied the topographical relationship of MIH - and CHH-gene-expressing perikarya in the MTXO in more detail [19].

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