Crustacean hyperglycaemic hormone (CHH)-like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes

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About 24 intrinsic neurosecretory neurons within the pericardial organs (POs) of the crab *Carcinus maenas* produce a novel crustacean hyperglycaemic hormone (CHH)-like peptide (PO-CHH) and two CHH-precursor-related peptides (PO-CPRP I and II) as identified immunochemically and by peptide chemistry. Edman sequencing and MS revealed PO-CHH as a 73 amino acid peptide (8630 Da) with a free C-terminus. PO-CHH and sinus gland CHH (SG-CHH) share an identical N-terminal sequence, positions 1–40, but the remaining sequence, positions 41–73 or 41–72, differs considerably. PO-CHH may have different precursors, as cDNA cloning of PO-derived mRNAs has revealed several similar forms, one exactly encoding the peptide. All PO-CHH cDNAs contain a nucleotide stretch coding for the SG-CHH$^{41–76}$ sequence in the 3′-untranslated region (UTR). Cloning of crab testis genomic DNA revealed at least four CHH genes, the structure of which suggest that PO-CHH and SG-CHH arise by alternative splicing of precursors and possibly post-transcriptional modification of PO-CHH. The genes encode four exons, separated by three variable introns, encoding part of a signal peptide (exon I), the remaining signal peptide residues, a CPRP, the PO-CHH$^{1–40}$/SG-CHH$^{1–40}$ sequences (exon II), the remaining PO-CHH residues (exon III) and the remaining SG-CHH residues and a 3′-UTR (exon IV). Precursor and gene structures are more closely related to those encoding related insect ion-transport peptides than to penaeid shrimp CHH genes. PO-CHH neither exhibits hyperglycaemic activity *in vivo*, nor does it inhibit Y-organ ecdysteroid synthesis *in vitro*. From the morphology of the neurons it seems likely that novel functions remain to be discovered.

Key words: alternative splicing, immunocytochemistry, neuropeptide, neurosecretion.

INTRODUCTION

Crustacean hyperglycaemic hormones (CHHs) from the X-organ sinus gland (SG) neurosecretory system in the crustacean eyestalk are involved in the regulation of blood glucose and lipids, hepatopancreatic enzyme secretion, Y-organ ecdysteroid production and gill ion transport [1,2]. After the first identification of a SG-CHH and its precursor mRNA in the green shore crab *Carcinus maenas* about 10 years ago, over 20 SG-derived CHHs have been isolated and identified, which in some animals even exist as multiple isoforms (e.g. up to six in penaeid prawns; for reviews see [1,3,4]). Furthermore, several CHH-precursor-related peptides (CPRPs) encoded by SG-CHH precursors of crab (*C. maenas* [5,6]), crayfish (*Orconectes limosus* [6,7]), lobster (*Homarus americanus* [6,8]) and penaeid shrimp (*Metapenaeus ensis* [9,10]) species have been isolated and Edman-sequenced, or their sequences have been deduced from the precursor. These peptides are obviously co-released with SG-CHHs, as has been demonstrated for the crab [11], but the functional significance of these peptides is still unclear. Recently, evidence has been provided in *M. ensis* for the existence of several genes arranged in clusters that give rise to precursors of different isoforms of SG-CHHs [9,10]. Similar gene structures have been revealed for another shrimp, *Penaeus monodon* [12]. Moreover, CHH-like peptides of structures and precursors similar to those of the decapod crustaceans occur in insects, where they are known as ion-transport peptides (ITPs [13–15]).

Whereas earlier preliminary data suggested the presence of CHH-like immunoreactivity or mRNAs in extra-eyestalk locations in several decapod crustaceans [3,16,17], only recently have CHH-immunoreactive cells been demonstrated immunocytochemically in the pericardial organs (POs) of *C. maenas* [18] and in the second roots of the lobster *H. americanus*. The latter is possibly the source of substances immunoreactive to an antiserum to *H. americanus* CHH in the haemolymph of long-term eyestalk-ablated lobsters [19,20]. In addition, another source of a transiently expressed CHH identical to that of the SG-CHH has recently been found in gut paraneurons of *C. maenas* which is involved in the control of ecdysis [11].

In this paper, we report on the identification by peptide chemistry of a novel CHH-like peptide and CPRPs expressed in immunocytochemically identified peripheral neurosecretory cells

Abbreviations used: CHH, crustacean hyperglycaemic hormone; CPRP, CHH-precursor-related peptide; MALDI-TOF MS, matrix-assisted laser desorption ionization–time-of-flight MS; MT, medulla terminalis; TFA, trifluoroacetic acid; SG, sinus gland; ITP, ion-transport peptide; PO, pericardial organ; Spe-, S-pyridylethylated; EP-AspN, endoproteinase AspN; CID, collision-induced dissociation; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase PCR; MIH, moult-inhibiting hormone; RP-HPLC, reversed-phase HPLC; UTR, untranslated region.

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in the POs of *C. maenas* (PO-CHH and PO-CPRPs) and report on the structural elucidation of multiple CHH genes coding for precursor products that are presumably modified at post-transcriptional or post-translational levels. Attempts to identify functions of PO-CHH have shown that it is, unlike the SG-CHH, neither hyperglycaemic nor active in inhibition of ecdysteroid production of crab Y-organs.

EXPERIMENTAL

Animals and tissue preparation

Specimens of green shore crabs *C. maenas* L. were caught by local fishermen at Yerseke, The Netherlands, or from the shore off the Isle of Anglesey, Wales, U.K., and maintained in recirculating seawater systems at 12–15 °C under a light/dark regime of 16 h:8 h. POs and SGs from crabs anaesthetized on ice were quickly dissected under ice-cold saline [21] and transferred into Eppendorf tubes (snap frozen in liquid N_2) or a fixative solution [22].

Immunochemical techniques

Whole-mount immunohistochemistry of CHH-immunoreactive structures in crab POs fixed overnight in phosphate-buffered paraformaldehyde/picric acid solution [22] was performed using established protocols, and immunofluorescent cells and terminals were visualized using FITC staining [23]. The only modification of the protocols was the use of 0.1 M Tris/HCl-buffered saline containing 0.5 % (v/v) Triton X-100 (pH 7.4) instead of PBS. A dot-immunobinding assay [23] was used to identify immunopositive reversed-phase HPLC (RP-HPLC) fractions. Antisera used were anti-*Carcinus* SG-CHH (code T1B1/4, 1:2000 final dilution [24]) and anti-*Carcinus* CPRP (1:3000 final dilution [11]). Two other antisera against a synthetic C-terminal hendecapeptide of the novel PO-CHH (code CtPOCHH-T6B1/3 or CtPOCHH-T7B1/3; both used at 1:3000 final dilution), extended N-terminally by a cysteine, which was covalently conjugated to maleimidated keyhole limpet haemocyanin (KLH [25]), were produced by two injections of 0.5 mg of KLH conjugate each into two Belgian giant rabbits within a 2 month period (methodology as in [24]). Preabsorption of antisera overnight with appropriate RP-HPLC-purified antigens (SG-CHH, PO-CHH or CPRP, 1 nmol calculated per 1 μl of crude antiserum) added to the final dilutions abolished immunostainings completely.

Peptide chemistry

750 POs extracted in batches of 50 in ice-cold 2 M acetic acid were purified by RP-HPLC on a Phenyl column (Waters μBondapak, 4.6 mm × 250 mm) followed by RP-HPLC on a Bakerbond C_{18} column (Mallinckrodt Baker, wide-pore, 4.6 mm × 250 mm) or on a Phenomenex Jupiter C_{18} column (Phenomenex, 5 μm particle size, 300 Å pore size, 4.6 mm × 250 mm) using linear water/acetonitrile/trifluoroacetic acid (TFA) gradients (hereon referred to as acetonitrile/TFA gradients) as described previously for crab SG extracts [26]. Peptides identified by dot-immunobinding assay were rechromatographed on the C_{18} column using a step gradient: 18–28.8 % (v/v) acetonitrile/0.1 % (v/v) TFA in 10 min, 28.8 % acetonitrile/TFA isocratic for 8 min and 28.8–31.2 % acetonitrile/TFA in 70 min (flow rate, 0.9 ml/min). Peptide fragments were generated from either native or reduced and S-pyridylethylated (Spe- [27]) peptides (1–1.5 nmol) using slightly modified methods described previously [26,28] by applying trypsin or endoproteinase AspN (EP-AspN, EC 3.4.24.33; both sequencing grade from Boehringer), with enzyme/substrate ratios of 1:25–30 (1:15 in the case of CPRPs) or 1:100, respectively, for 15–18 h at 37 °C. SG-CPRP obtained from crab SGs was used for reference. Fragments were RP-HPLC-purified on the Phenyl or C_{18} columns. Standard peptide synthesis using Fmoc (9-fluorenylmethyloxy-carbonyl)-derivatized amino acids was done on an Applied Biosystems model 433A synthesizer. Peptides for bioassaying were quantified by amino acid analysis on RP-HPLC according to either *o*-phthaldialdehyde [29] or Fmoc-chloroformate [30] pre-column derivatization methods.

MS and sequencing

Small amounts of peptides and fragments (one-fiftieth of a sample, approx. 5–20 pmol) were first analysed by matrix-assisted laser desorption ionization–time-of-flight MS (MALDI-TOF MS) on a VG Tofspec SE equipped with a N_2 laser (337 nm; Micromass, Manchester, U.K.) operating in linear (acceleration voltage, 25 kV) and/or reflectron mode (acceleration voltage, 20 kV; reflectron voltage, 28.5 kV) at laser energies adjusted for optimal resolution and signal/noise ratios. Final spectra were plotted from averaged results of 10–20 shots. Fractions were analysed further and/or sequenced by nanoflow ESI-Qqoa-TOF MS (electrospray ionization double-quadrupole orthogonal-acceleration time-of-flight MS) on a Q-TOF system (Micromass) as described elsewhere [31]. Sequences were derived using MS/MS or tandem MS by analysing fragment ions generated from a selected precursor ion by collision-induced dissociation (CID). In order to enhance or equalize the efficiency of peptide ion fragmentations, the collision energy was typically varied between 20 and 35 V. In addition, Edman sequencing of approx. one-tenth to one-half of a sample was performed on a Beckman LF 3000 automated gas-phase sequencer, or on an Applied Biosystems Procise 492 microsequencer running in pulsed-liquid mode.

Bioassays and release experiments

Haemolymph glucose bioassays were performed essentially as described in [28] using RP-HPLC-purified and quantified peptide samples from PO or SG extracts for injection. Haemolymph was taken from the hypobranchial sinus every 30 min from 0 to 3 h. In other experiments haemolymph samples were taken at 0 and 2 h after injection. PO-CHH and SG-CHH were tested by *in vitro* bioassay for the inhibition of ecdysteroid synthesis of isolated crab Y-organs and for stimulation of cGMP production in isolated Y-organ and heart tissues as described earlier [32,33]. Furthermore, *in vitro*-release experiments were carried out on 10 freshly dissected POs following regimes described previously [34] using a crab saline [21] with a 10-fold molar excess of KCl and an equivalent molarity of NaCl subtracted. Substances released into high K^+ salines and the subsequent normal wash salines were combined from three successive release incubations, desalted on SepPak® cartridges (Waters), eluted with 60 % (v/v) aqueous acetonitrile containing 0.1 % (v/v) TFA, and dried in a vacuum centrifuge (SpeedVac, Savant). Samples (10 %) were subjected to MALDI-TOF analysis.

RNA preparation and cDNA synthesis

Total RNA was isolated with Trizol® (Gibco-BRL Life Technologies) following the manufacturer's instructions. The mRNA was isolated from total RNA preparations with the OligoTex kit (Qiagen). First-strand cDNA was synthesized from total RNA (2 μg) with 200 units of Moloney-murine-leukaemia virus reverse transcriptase (Superscript, Gibco-BRL) according to the manu-

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facturer's protocol for 1 h at 45 °C in a final volume of 20 μl in presence of a ribonuclease inhibitor (2 units, Stratagene) and either an oligo-dT anchor primer (Roche Diagnostics) for 3′-RACE (rapid amplification of cDNA ends) or a gene-specific primer (AS2, see the PCR section and Figure 7) for 5′-RACE. cDNA synthesis was stopped by incubating the sample at 70 °C for 10 min and residual RNA was digested with 2 units of RNAse H (Stratagene) for 20 min at 37 °C. After a final heat-inactivation step (70 °C, 10 min), the cDNAs were purified on High-Pure columns (Roche Diagnostics) and eluted in 50 μl of 10 mM Tris/HCl, pH 7.5.

Preparation of genomic DNA, restriction digestion and Southern blotting

Genomic DNA was extracted with phenol from the testis of a single crab following standard procedures [35]. The isolated genomic DNA was 40–150 kb in size. For Southern blotting, 10 μg of genomic DNA were digested overnight with the enzymes detailed below in the appropriate reaction buffer at 37 °C. The restriction fragments were separated on a 0.7% (w/v) agarose gel in 0.04 M Tris/acetate buffer, pH 8.0, containing 0.001 M EDTA, and transferred to positively charged nylon membranes (Roche Diagnostics). The membrane was probed with a digoxygenin-labelled DNA corresponding to nucleotides 15–538 of the PO-type CHH cDNA (see Figure 4). Prehybridization, overnight hybridization at 42 °C, stringency washes and chemiluminescence detection (Dig Chemiluminescence kit with CSPD-star, Roche Diagnostics) of labelled fragments were performed according to the manufacturer's protocol.

Northern hybridization

The mRNA isolated from 5 μg of total RNA of crab POs or medullae terminales (MTs), and another 10 μg of total RNA from POs were subjected to electrophoresis on a 1.2% (w/v) agarose/formaldehyde gel according to standard procedures [35]. The gel was rinsed briefly in water and 10 × SSC buffer, and the RNA was transferred to a positively charged nylon membrane by overnight capillary blotting. The blot was probed with a digoxygenin-labelled DNA corresponding to nucleotides 15–301 (see Figure 4). The hybridization protocol was essentially the same as detailed for the Southern blotting except that prehybridization and hybridization were performed at 50 °C.

PCR, cloning and sequencing

PCR reactions were performed in 25 μl samples with 1 unit of a proofreading DNA polymerase (Expand® from Roche, or *Pfu* from Stratagene), and 10 pmol of each primer in the appropriate reaction buffer containing 0.2 mM dNTPs and 1.5 mM $MgCl_2$. Seven different primers have been used in this study: S1 [P1, 5′-TCGCAGAAGGAAGACGTACACCTCCTCC-3′, common 5′-untranslated region (UTR) of exon I], S2 (C2f, 5′-CGACAC-GTCCTGCAAGGGTG-3′, on exon II), S3 (PO1f, 5′-ACCTC-CTATGTTGCCTCGGC-3′, exon II), AS1 (C2r, 5′-CACCCT-TGCAGGACGTGTCG-3′, exon II), AS2 (Pospecrev, 5′-TAA-GTCCATCCCTGCTGCG-3′, PO-CHH-specific, exon III), AS3 (PO2r, 5′-AGTTGCTATAGCAGTTTGAT-3′, exon IV) and AS4 (M1, 5′-AATTATGTCGCCTCCTAAAT-3′, long 3′-UTR of exon IV). For initial reverse transcriptase PCR (RT-PCR) steps 1 μl of the purified cDNA was used as template. For subsequent nested-PCR steps 1 μl of a 1:100 dilution from the initial PCR reaction served as template. Conditions in the RT-PCR experiments consisted of an initial denaturation step at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 1–3 min depending on the expected size of the amplicon. PCR amplification of genomic DNA was performed on 500 ng of genomic DNA as template. The DNA polymerase was added after an initial denaturation step for 10 min at 95 °C (hot start). PCR cycles were essentially the same as for RT-PCR except that the last 20 cycles contained an extension of the elongation step of 10 s/cycle.

PCR products were either purified by extraction from agarose gels by the Geneclean method or by spin-column purification (High-Pure). A 3′ A-overhang was generated on the purified DNA by incubation with dATP (0.2 mM) and *Taq* polymerase (2.5 units) in PCR reaction buffer containing 15 mM $MgCl_2$ for 30–60 min at 72 °C. Subsequently, DNA was cloned either using a TOPO-TA vector (Invitrogen) or pGemT-easy (Promega) according to the manufacturers' instructions. Bacterial transformations were plated on LB/ampicillin agar and recombinant clones were picked and grown in liquid media. After plasmid minipreps, the insert size was analysed by restriction digest and agarose gel electrophoresis. Recombinant plasmids carrying inserts of the expected size were subjected to automated DNA sequencing (MWG, Martinsried, Germany; Eurogentec, Seraing, Belgium; or Agowa, Berlin, Germany).

RESULTS

Peptide localizations

Immunocytochemistry of crab POs revealed immunopositive intrinsic multipolar neurons with neurohaemal release terminals abutting the surface of segmental nerves, anterior and posterior bars and, preferentially, the ventral trunks. Up to four neurons

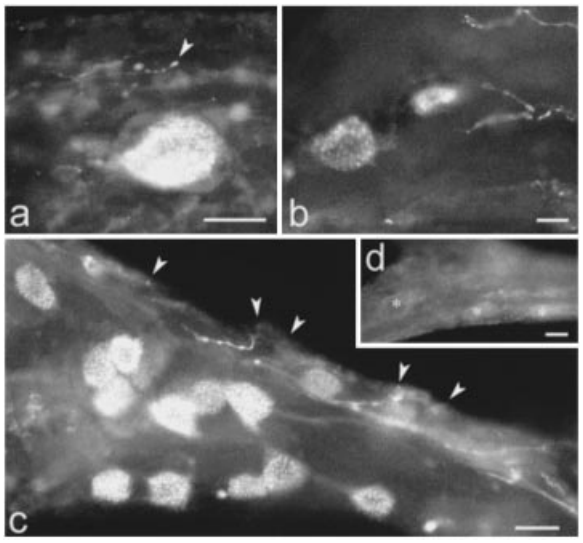

Figure 1 Intrinsic neurosecretory neurons and terminals in the POs of the crab *C. maenas*

(**a**) Multipolar neuron in the anterior bar stained by anti-SG-CHH; single terminal at the surface of the bar (arrowhead). (**b**) Two neurons in the anterior bar close to the ventral trunk labelled by anti-SG-CPRP. (**c**) 13 neurons in the posterior bar labelled by PO-CHH C-terminus-specific antiserum (photomontage of two focal planes); note the terminals at the surface of the ventral trunk (arrowheads) arising from branching varicose fibres. (**d**) Pre-absorption control of a similar posterior bar region to that in (**c**); the asterisks show unlabelled cells. Whole-mount FITC-immunofluorescence preparations are shown, observed with a Zeiss Axioskop fluorescence microscope; scanned colour slide micrographs were grey-scaled and assembled with CorelDraw version 7.0. Scale bars, 50 μm.

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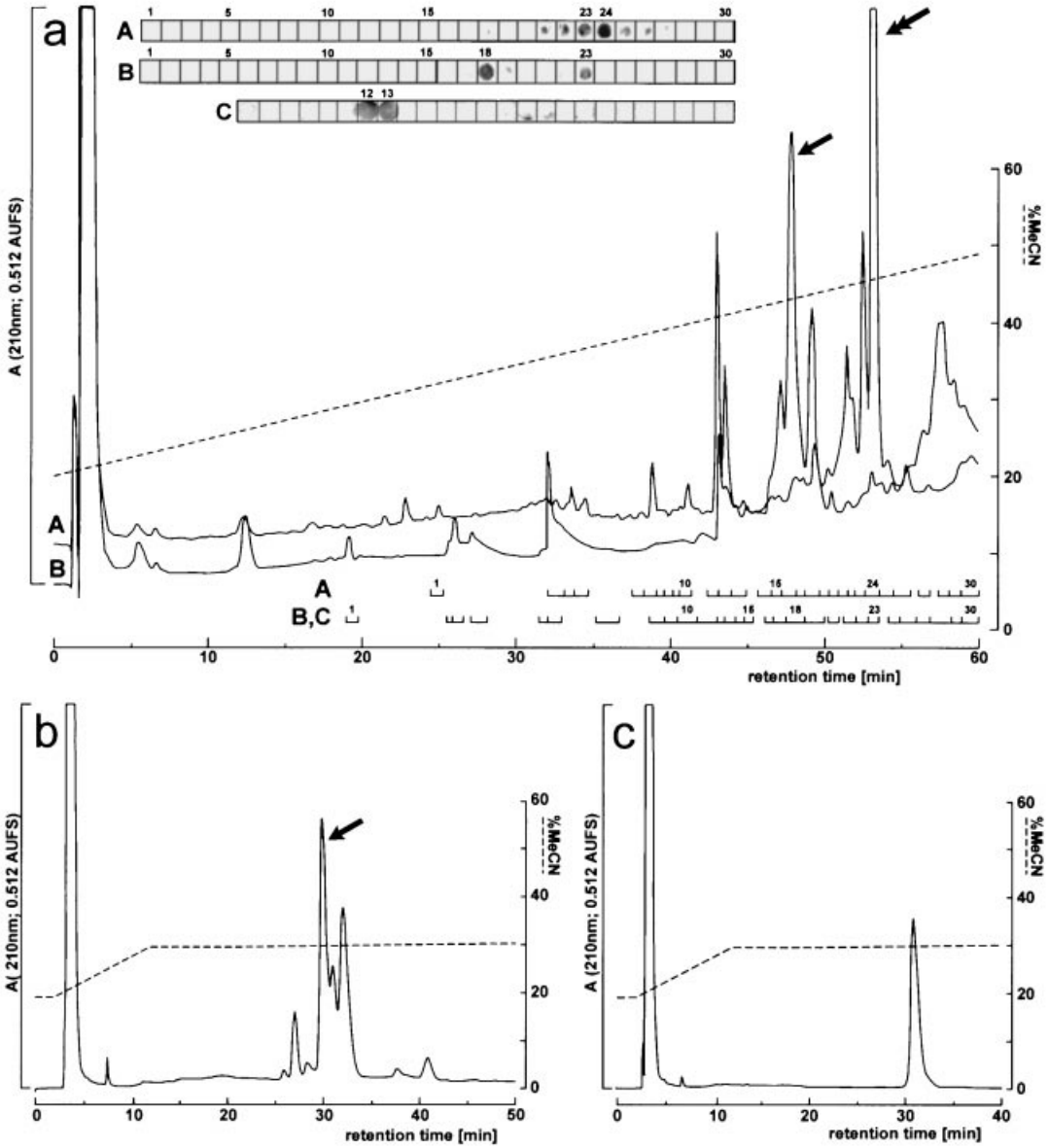

Figure 2 RP-HPLC purification of PO-CHH from the crab *C. maenas*

(**a**) Comparison of manually collected fractions after RP-HPLC of extracts from 20 POs and 23 SGs by dot-immunobinding assay using anti-SG-CHH shows that the main SG-CHH (double arrow; fraction 24, lane and panel A) elutes about 7 min later than the CHH-immunoreactive peptide from POs (arrow, fraction 18, lane and panel B). CPRP-immunoreactive RP-HPLC fractions from PO extracts (fractions 12 and 13; lane and panel C) elute at the same retention times as those from SG extracts. (**b**) Rechromatography of the CHH-immunoreactive fraction from approx. 180 POs containing PO-CHH (arrow). (**c**) Third and final purification step of the PO-CHH (approx. 180 PO equivalents). MeCN, acetonitrile.

occur in the anterior bar and 15–20 neurons in the posterior bar (Figure 1). The polyclonal anti-SG-CHH and anti-SG-CPRP antisera and the PO-CHH C-terminus-specific antisera gave the same staining patterns, but the latter often produced more intense and consistent stainings.

PO-CHH and PO-CPRP identification

After immunocytochemistry using the polyclonal anti-SG-CHH and anti-SG-CPRP, we first assumed that both the CHH and CPRP peptides may be identical in SG and PO. Therefore, our strategy was to identify peak fractions obtained after RP-HPLC of extracts from both neurohaemal organs by dot-immunobinding assay using the same antisera. In the case of the PO extracts, a prominent anti-SG-CHH immunopositive fraction eluted about 7 min earlier than that of the known SG-CHH (Figure 2a). This fraction contained a novel CHH-like peptide, tentatively named PO-CHH, that was purified by two RP-HPLC rechromatography steps again combined with dot-immunobinding (Figures 2b and 2c). MS including CID analysis and Edman sequencing performed on overlapping proteolytic fragments of Spe-PO-CHH, native PO-CHH and Spe-SG-CHH (Table 1) obtained after tryptic and EP-AspN digestion unambiguously revealed the sequence of a 73 amino acid peptide with a N-terminal pyroglutamate and C-terminal carboxyl group (Figure 3). It had a mass of 8630.81 ± 0.3 Da (Q-TOF, $M+H^+$; calculated mass, 8630.5 Da). Since digestion with carboxypeptidase Y proved impossible, the structure of the C-terminal

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Table 1 MS and sequence data of tryptic (a) and EP-AspN-generated (b) fragments of Spe-SG-CHH and Spe-derivatized or native PO-CHH of *C. maenas*

The peptide sequences were obtained by Edman degradation (bold type) or by CID/Q-TOF sequence analysis (underlined). Peak P7 has been repeatedly sequenced from two independent batches of animals. Ph and C_{18}, obtained after RP-HPLC on Phenyl or C_{18} columns respectively.

(a) Tryptic fragments of Spe-SG-CHH (T-series) and Spe-PO-CHH (P-series)

Proteolytic fragment	Peak	Retention time (min)	Mass (Da, calculated average, $M+H^+$)	Mass (Da, calculated mono-isotopic, $M+H^+$)	Mass (Da, MALDI-TOF, $M+H^+$)	Mass (Da, Q-TOF, $M+H^+$)
pEIYDTSCK	T4 = P3 (C_{18})	22.3	1046.20	1045.76	1045.5	1045.8
GVYDR	T1 = P1 (C_{18})	13.3	609.66	609.30	609.3	609.36
ALFNDLEHVCDDCYNLYR	T6 = P5 (C_{18})	40.6	2414.76	2413.54	2410.6	2413.25
TSYVASACR	T3 = P2 (C_{18})	19.1	1063.24	1062.74	1061.9	1062.45
NNCFENEVFDVCVYQLYFPNHEEYLR	P7 (Ph)	44.7	3496.94	3495.02	3494.3	3495.82
DGLKG(-OH)	P0C (C_{18})	9.6	488.53	488.26	–	488.54

(b) EP-AspN fragments of Spe-derivatized or native (n) PO-CHH series, and cystine-coupled tryptic fragments (last 3 peptides)

Proteolytic fragment	Peak	Retention time (min)	Mass (Da, calculated average, $M+H^+$)	Mass (Da, calculated mono-isotopic, $M+H^+$)	Mass (Da, MALDI-TOF, $M+H^+$)	Mass (Da, Q-TOF, $M+H^+$)
DTSCKGVY*	A3	18.8	888.97*	888.83*	–	888.35*
DRALFN	A9, A6n	27.0	735.82	735.38	734.7	735.39
DLEHVC*	A6	22.3	731.80*	731.30*	–	731.37*
DLEHVCD	A7	22.7	936.05	935.63	934.0	–
DDCYNLYRTSYVASACRNNCFENEVF	A12, A10 (Ph)	37.0	3413.85	3411.75	3410.9	3413.37
DDCYNLYRTSYVASACRNNCFENEVF	A11n	39.9	3098.38	3096.29	3098.3	–
DDCYNLYRTSYVASACRNNCFEN	A10	31.2	3038.42	3036.57	3034.9	–
DDCYNLYRTSYVASACRNNCF	A11	32.9	2795.20	2793.49	2792.6	–
DCYNLYRTSYVASACRNNCF	A11	32.9	2680.11	2678.46	2677.5	–
DVCVYQLYFPNH	A13, A10 (Ph)	37.4	1603.86	1602.84	1639.9 ($M+K^+$)	1602.98
DVCVYQLYFPNHEEYLRSRDGLKG	A11n	39.9	2903.24	2901.39	2904.7	–
DVCVYQLYFPNHEEYLRSR	A14	37.6	2537.88	2536.30	2534.8	–
EEYLRSRDGLKG-OH	A8, A3n	24.6	1423.57	1422.73	1422.7	1422.99
EEYLRSR	A4, A2n	20.0	953.04	952.48	953.3	–
DGLKG(-OH)	A1n	10.7	488.53	488.26	–	488.46
PO-CHH$^{4–11}$ + PO-CHH$^{18–23}$ + PO-CHH$^{24–61}$	A13n	42.6	6159.77	6156.60	6162.5	–
PO-CHH$^{18–23}$ + PO-CHH$^{24–49}$	A11n	39.9	3811.16	3808.57	3811.6	–
DTSCK NNCFENEVF (C^7–C^{43})		32.1	1666.79	1665.64	1664.4	–
DLEHVC TSYVASACR (C^{23}–C^{39})		26.7	1670.86	1669.73	1669.7	–
DDCYNLYR DVCVYQLYFPNH (C^{26}–C^{52})		39.6	2557.83	2556.09	2553.1	–

* Sulphoxidized cysteine residue detected instead of Spe-Cys.

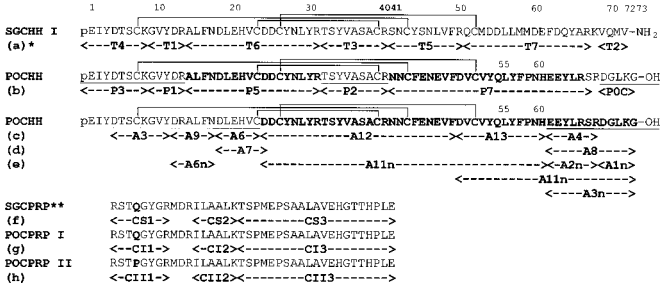

Figure 3 Sequences and fragments of SG-CHH [26], PO-CHH, SG-CPRP [6] and PO-CPRPs

RP-HPLC-separated and MS-detected tryptic (T in **a**; P in **b**; C in **f**–**h**) and EP-AspN-generated (A in **c**–**e**) fragments (arrows) of Spe-SG-CHH I (**a**; * = found identical to [26]) and Spe-PO-CHH on a Phenyl (**c**) and C_{18} (**c**, **d**) columns, or of native PO-CHH (**e**) separated on a C_{18} column. CID/Q-TOF-(underlined) and Edman-sequenced (bold) fragments are indicated. Note that the first 40 amino acids of SG-CHH and PO-CHH are identical but the rest are largely different; note also the amino acid exchange in position 4 of PO-CPRP II versus the identical sequences of SG-CPRP (**f**; ** = according to [5,6]) and PO-CPRP I; PO-CPRP sequences were deduced from the mRNA precursors and tryptic fragments (**g**, **h**; see Figure 4 and the text).

pentapeptide DGLKG-OH was confirmed by CID, Edman-sequence analysis and peptide synthesis. Retention-time analysis by RP-HPLC on the C_{18} column revealed that a synthetic presumptive DGLKamide eluted about 2 min earlier than the native or the synthetic fragment DGLKG-OH (results not shown). The first 40 residues of this PO-CHH are identical to those of SG-CHH but the remaining 33 are very different. The PO-CHH fragment analysis clearly showed that EP-AspN not only cleaves at preferred D residues but also at various E residues. This yielded two peptides, EEYLRSR and, in low amounts, EEYLRSRDGLKG-OH, the latter providing the missing but decisive overlap with the three C-terminal tryptic fragments P7, the dipeptide SR (not found after RP-HPLC) and DGLKG-OH, as confirmed by Q-TOF sequencing; Table 1, Figures 2a and 3). To confirm the residues Q^{55} and N^{60}, for reasons detailed in the section on cDNA cloning and sequencing, Edman sequencing was performed twice on fragment P7 and once on fragment A13 (see Table 1) after preparation of all three samples from different batches of animals.

For the assignment of disulphide bridges, one of the RP-HPLC-separated EP-AspN-generated fragments of native PO-CHH was further cleaved by trypsin, rechromatographed and subjected to mass analysis and amino acid analysis. This late-eluting peak (A13n, 42.6 min, C_{18} column, Table 1) contained two incompletely cleaved RP-HPLC-inseparable EP-AspN fragments of the same mass, 6162.5 Da, as measured by MALDI-TOF MS, which consisted of the peptides A3, A6 and PO-CHH$^{24–61}$, and A3, PO-CHH$^{18–49}$ and PO-CHH$^{50–61}$, both coupled

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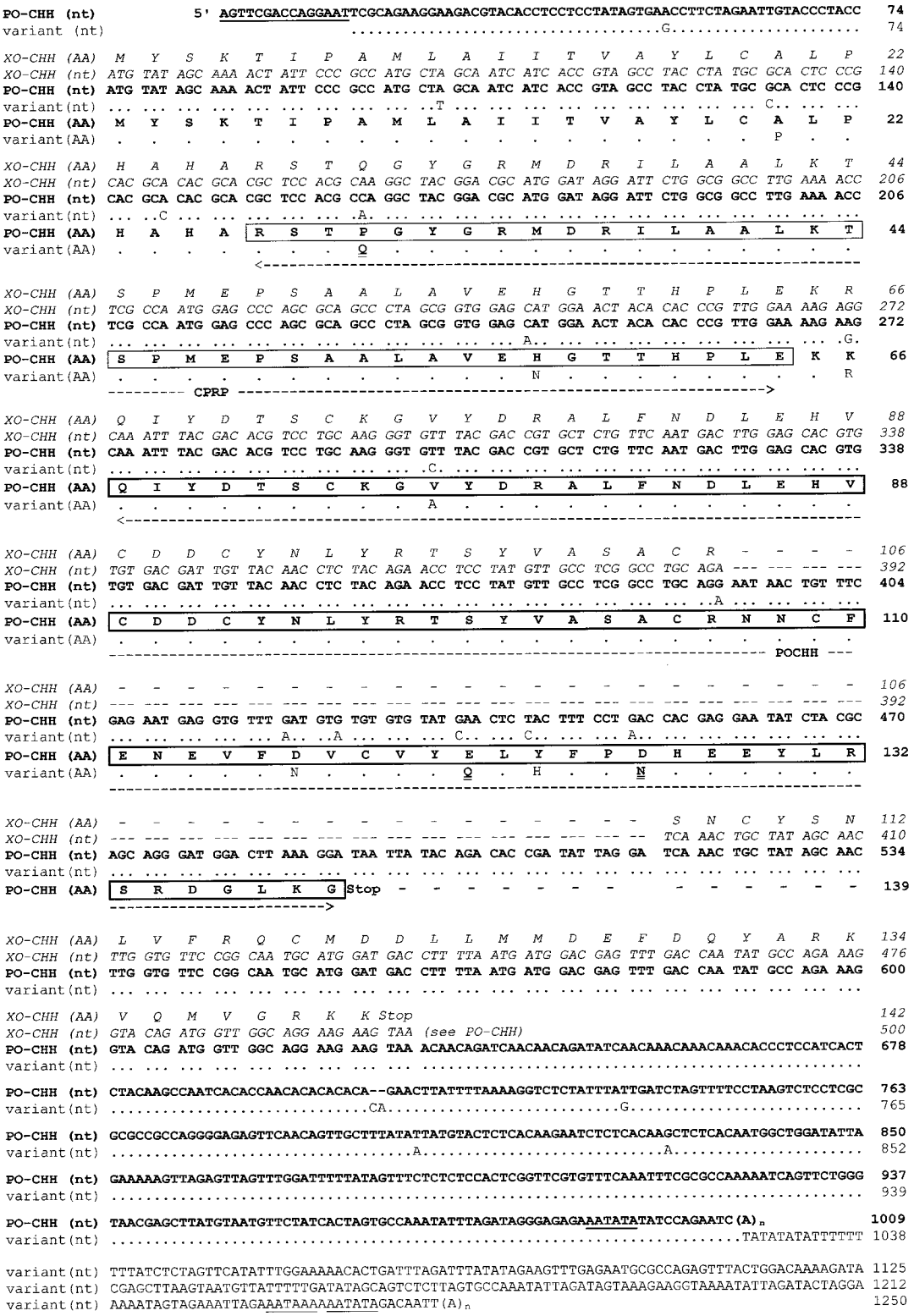

Figure 4 Sequence alignment of PO-CHH and SG-CHH cDNAs of *C. maenas*

Aligned nucleotide and deduced amino acid (AA) sequences of a full-length PO-type CHH cDNA and the compiled SG-type CHH cDNA found in this study. All observed nucleotide variants and their deduced translation products found in at least two different cDNA clones are summarized in the variant lines. The mature PO-CHH and CPRP peptide sequences are boxed and labelled. The 5′ nucleotides detected by 5′-RACE only, putative polyadenylation signals (canonical AATAAA and variant AATATA) are underlined. Note the variants encoding exactly the PO-CHH and CPRP peptide sequences obtained by peptide chemistry showing the PO-CHH Q^{55} and N^{60} residues (precursor positions 121 and 126), and the CPRP P^4 and Q^4 residues (precursor position 30), respectively (double underlined; GenBank accession numbers AF286084 and AF286092).

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via intact disulphide bridges (calculated mass, 6159.77 Da). Tryptic fragmentation of this peak fraction yielded four peptides, the 'non-decisive' DTSCK + A6 + PO-CHH$^{31–49}$ (3313.7 Da; calculated mass, 3318.61 Da), a fragment of the latter EP-AspN fragment, and three other peptides, each consisting of two peptides covalently linked by a single disulphide bridge (Table 1, last three peptides) arising from the former EP-AspN fragment. Within the limits of accuracy of the MALDI-TOF instrument used, this procedure proved that the disulphide bridges of PO-CHH have the same configuration as that known from SG-CHH [26,28].

Rechromatography (C_{18} column, linear gradient of 27–33 % acetonitrile/TFA in 45 min, 1 ml/min flow rate) of the anti-CPRP immunopositive peak fractions eluted in the first-step RP-HPLC (43.1 and 43.5 min in Figure 2a) revealed three different peptides, the third one occurring in much smaller amounts ($\approx$ 5 % of total peak areas) than the other two. Fragmentation and MS analyses of two PO-CPRPs compared with a standard of the known SG-CPRP revealed PO-CPRP I and II with molecular masses and amino acid compositions fitting exactly the 38-residue peptide sequences deduced from different mRNA precursors (Figures 3 and 4). Amino acid analysis (*o*-phthaldialdehyde method) was performed on three tryptic fragments each of SG-CPRP (fragments CS1–CS3) and PO-CPRP I (fragments CI1–CI3) and PO-CPRP II (fragments CII1–CII3; obtained after RP-HPLC on a C_{18} column eluted with a linear gradient of 18–48 % acetonitrile/TFA in 60 min at a flow rate of 1 ml/min). Retention times (in min) and amino acid compositions (numbers of fragment amino acids in brackets calculated in relation to amino acid standards) were almost identical for the first fragments [CS1 (14.8 min)/CI1 (14.8 min), Glx (1.34/1.63), Ser (0.67/1.03), Gly (2.48/2.79), Thr (0.91/1.02), Arg (2.25/2.03) and Tyr (1.08/1.01)], but differed in the case of CII1 [19.57 min, Glx (none), Ser (1.19), Gly (2.90), Thr (0.85), Arg (2.0) and Tyr (1.0)]. The second and third fragments of all three CPRPs had almost identical retention times and amino acid compositions [CS2 (35.73 min)/CI2 (35.49 min)/CII2 (35.53 min), Ala (2.2/2.23/2.4), Ile (0.9/0.84/0.74), Leu (2.1/2.24/2.16) and Lys (1.09/1.16/1.17); and CS3 (40.25 min)/CI3 (40.39 min)/CII3 (40.46 min), Glx (3.5/3.58/3.49), Ser (1.92/1.6/1.82), His (2.21/2.09/2.31), Gly (0.97/0.85/0.93), Thr (2.29/2.02/2.22), Ala (3.63/3.38/3.32), Met (0.96/0.65/0.95), Val (1.04/1.01/1.05) and Leu (2.39/2.16/2.2)]. Q-TOF analyses confirmed that PO-CPRP I is identical to SG-CPRP (Figure 3f; calculated mono-isotopic mass, 4092.07 Da; measured mass, 4092.7 Da). PO-CPRP II had a mono-isotopic mass of 4061.9 Da (calculated mass, 4061.07 Da) and obviously differs only in position 4 (P^4 instead of Q^4; Figures 3g, 3h and 4). PO-CPRP III had a mono-isotopic mass of 4031.9 Da, but further structural data have not yet been obtained. This mass, however, is not identical to a CPRP-like peptide with amino acid exchanges in positions 4 (Q^4) and 31 (N^{31}; calculated mass, 4069.06 Da), the sequence of which was deduced from another cDNA clone (Figure 4).

Bioassays and release experiments

SG-CHH or PO-CHH (both 10 or 20 pmol) were injected either into eyestalk-ablated or into intact crabs in separate groups. For SG-CHH, this resulted in significant increases (2–5 fold) in haemolymph glucose levels compared with controls, as expected. However, increases in haemolymph glucose levels after PO-CHH injection were not observed, either by analysis of the time course of hyperglycaemia over 3 h (Figure 5), or by sampling after 2 h (20 pmol injected) in different sets of experiments. Co-injection of 10 pmol of SG-CHH with 50 pmol of PO-CHH also did not change the pattern of hyperglycaemia seen in animals injected with SG-CHH alone (results not shown). *In vitro* bioassays for testing inhibition of Y-organ ecdysteroid production and cGMP accumulation in Y-organs and heart tissues performed at different times of the year resulted in clear-cut effects for SG-CHH. PO-CHH had no significant effects on inhibition of Y-organ ecdysteroid production (Table 2), although it increased Y-organ cGMP levels significantly to a slightly lesser extent than SG-CHH, especially in animals in winter time (Table 3). However, PO-CHH had no effect on heart-tissue cGMP production in comparison with SG-CHH, which increased cGMP levels more than 20-fold (Table 3). MALDI-TOF analysis of an *in vitro* releasate evoked from POs by high-K^+ saline showed the occurrence of a large peak at the same molecular mass as that of PO-CHH, along with other much smaller peaks at lower masses.

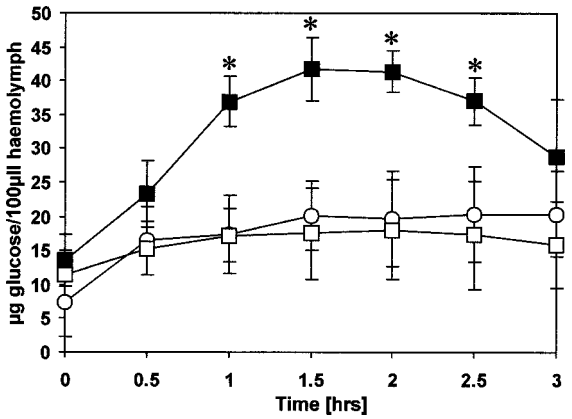

Figure 5 Haemolymph glucose bioassaying of SG-CHH and PO-CHH

Changes in haemolymph glucose levels of eyestalk-ablated crabs over 3 h of sampling every 30 min ($n = 6$; means ± S.D.) after injection of 10 pmol of RP-HPLC-purified SG-CHH (■), 10 pmol of PO-CHH (□) or saline (○). Asterisks indicate significant differences compared with controls ($P < 0.01$, Student's t test).

cDNA cloning and sequencing

A first RT-PCR approach, using primer S1 corresponding to 28 nucleotides in the 5′-UTR and primer AS3 corresponding to a 20 nucleotide stretch in the last third of the coding sequence of the known X-organ cDNA encoding the SG-CHH [5], was used to compare cDNAs from POs and MTs. Surprisingly, the major amplicon (approx. 500 bp) observed for PO cDNA was 150 bp larger than the major amplicon derived from MT cDNA (350 bp). A smaller fragment, which co-migrated with the major amplicon generated by RT-PCR from MT samples, was also always observed as a minor product after RT-PCR of PO cDNA. Likewise, MT cDNA gave rise to a minor PCR product of about 500 bp in size. Sequencing of the larger amplicon form of PO cDNA revealed a 512 bp nucleotide sequence with an open reading frame of 417 bp (Figure 4). The nucleotide and deduced amino acid sequences were almost identical to the SG-CHH cDNA for the 5′-UTR until nucleotides 363–365, coding for amino acid 40 of the mature CHH-like peptide. The following 102 bp in the PO-CHH cDNA, terminated by a stop codon, were significantly different from the SG-CHH cDNA. The open reading frame was followed by 23 nucleotides of the 3′-UTR preceding the reverse-primer binding site, which in the case of SG-CHH cDNA is located within the coding sequence. A subsequent PCR with the downstream primer AS2, specific for

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Table 2 ***In vitro*** **ecdysteroid production of crab Y-organs elicited by SG-CHH and PO-CHH**

Means ± S.E.M. in ng of ecdysteroids/Y-organ × 24 h; $n = 5$, in three different experiments (incubation with each peptide at a final concentration of 50 nM). *Significant difference between treatment and control ($P < 0.05$; Student's paired t test).

	Ecdysteroid production					
	SG-CHH			PO-CHH		
Experiment date	Control	SG-CHH treatment	% Inhibition	Control	PO-CHH treatment	% Inhibition
13/01/2000	13.6 ± 1.7	4.8 ± 0.4*	63.1 ± 4.1	23.0 ± 4.7	16.7 ± 2.4	21.1 ± 8.5
12/03/2000	14.9 ± 3.7	7.1 ± 1.0*	50.8 ± 6.0	16.9 ± 3.7	16.7 ± 4.6	3.6 ± 7.1
16/08/2000	12.9 ± 1.7	4.8 ± 1.0*	64.5 ± 3.6	15.4 ± 2.4	13.9 ± 2.2	8.3 ± 4.1

Table 3 ***In vitro*** **cGMP production of crab Y-organs and hearts elicited by SG-CHH and PO-CHH**

Data are means ± S.E.M. in terms of nmol of cGMP/organ (Y-organ or half of the heart); $n = 5$, in different experiments (incubation with each peptide at a final concentration of 50 nM). All treatments are significantly different from controls except for PO-CHH on heart tissue; *significant differences between treatments with different peptides ($P < 0.05$; Student's t test).

	cGMP production					
	SG-CHH			PO-CHH		
Experiment date	Control	SG-CHH treatment	Elevation ratio	Control	PO-CHH treatment	Elevation ratio
Crab Y-organ						
17/01/2000	1.2 ± 0.2	4.2 ± 0.7	3.8 ± 0.6	1.6 ± 0.4	4.0 ± 0.7	3.1 ± 1.1
18/01/2000	0.3 ± 0.0	1.8 ± 0.1	6.2 ± 0.7	0.5 ± 0.1	2.2 ± 0.3	5.0 ± 0.5
19/08/2000	1.2 ± 0.4	5.3 ± 0.9*	6.5 ± 1.8	0.8 ± 0.1	2.7 ± 0.3*	3.5 ± 0.4
Crab heart						
23/01/2000	7.4 ± 5.5	26.1 ± 2.1*	21.7 ± 8.3	1.4 ± 0.2	2.0 ± 0.4*	1.6 ± 0.4

the PO-CHH sequence, confirmed the expression of this newly detected CHH-like isoform in POs, and also showed low but detectable expression in MT tissue (results not shown).

The complete PO-CHH cDNA sequence was obtained after a 3′-RACE approach using primer S1 as upstream primer. The cloned amplicons (between 1000 and 1200 bp in length) contained a short stretch of the 5′-UTR, the total coding region, and a 3′-UTR ending in a poly(A)$^+$ tail. Application of 5′-RACE with primer AS2 revealed the missing base pairs of the 5′-UTR, which proved to be identical in all cloned products (Figure 4). In total, we amplified, isolated, cloned and sequenced more than 30 full-length and partial cDNAs from POs. A total of 17 sequences found in at least two clones from independent reverse translations have been submitted to the GenBank Nucleotide Sequence Database (accession numbers AF286078–AF286094). Of these, 13 cDNA clones were of the PO-CHH cDNA variant type whereas four clones lacked the coding region for PO-CHH$^{41–73}$ and corresponded to the SG-CHH-type cDNA. As summarized in Figure 4, cDNAs showed slight differences in base composition and length. Modifications were more frequently observed in the coding region for the signal peptide and the CPRP variants, which include the PO-CPRP I and II isoforms (Q^4 and P^4 at precursor position 30, respectively) found by peptide chemistry, rather than in the coding region for the mature PO-CHH. However, the conceptual translations indicated at least four different mature PO-CHH variant peptides. In particular, we found only two clones coding for the Q^{55} and N^{60} residues (precursor positions 121 and 126; GenBank accession numbers AF286084 and AF286092) corresponding to the PO-CHH peptide sequencing results.

In most 3′-RACE cDNA clones, the 3′-UTR was 512 bp long and contained the AATATA polyadenylation-signal variant 9 bp upstream of the poly(A)$^+$ tail and two ATTTA RNA-instability signals. Two clones, one PO-type and one SG-type, terminated in a 757 bp-long 3′-UTR with a canonical polyadenylation signal 17 bp upstream of the poly(A)$^+$ tail and contained two additional ATTTA copies. Compared with the previously described SG-CHH cDNA, both forms of the 3′-UTR have to be considered as truncated, since the 3′-UTR has a total length of 1.3 kb, contains a classical polyadenylation signal 11 bp upstream of the poly(A)$^+$ tail and displays a total of 10 copies of the ATTTA RNA-instability motif. In particular, all cDNAs found in this study lacked a long CT repeat first described for the 3′-UTR of the SG-CHH precursor (GenBank accession number X17596). However, RT-PCR of PO cDNA with primer combinations corresponding to the long 3′-UTR always yielded an unambiguous amplicon of the expected size, thus suggesting that the long form of the 3′-UTR also exists in POs. In Northern-blotting experiments (Figure 6), we exclusively detected a single hybridizing band of about 2 kb, which showed no detectable difference in size from the signal obtained with mRNA from MTs.

Genomic organization

The frequent modifications observed in the cDNAs indicated the existence of multiple gene copies. Moreover, the fact that the coding sequence for SG-CHH$^{41–76}$ was invariantly found in the 3′-UTR of the PO-CHH cDNAs suggested tissue-specific differential splicing of CHH pre-mRNAs. These assumptions were confirmed by restriction digest followed by Southern blotting (Figure 7a). Single-enzyme digestions always resulted in at least four hybridizing bands, thus indicating the existence of multiple gene copies. Combined *Xba*I/*Spe*I digestion that would generate an invariant 900 bp restriction fragment for all observed

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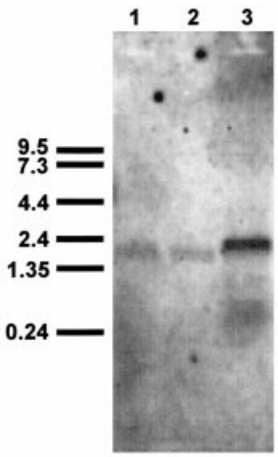

Figure 6 Comparative Northern-blot analysis

CHH gene transcripts found in crab POs and MT (including the X-organ). Lane 1, mRNA (5 ng) from POs; lane 2, mRNA (5 ng) from MT; lane 3, total RNA (10 μg) from POs. Marker lengths are shown in kb. Note that the only hybridizing mRNA product in both tissues was about 2 kb in length.

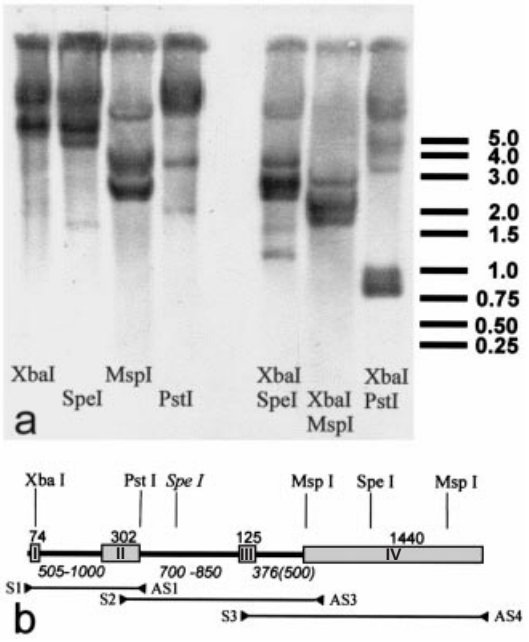

Figure 7 Restriction analysis of *C. maenas* CHH genes

(**a**) Southern blot of genomic DNA from crab testis after restriction digest with the enzymes indicated, blotted on to a nylon membrane and hybridized with a Dig-labelled DNA probe corresponding to nucleotides 15–538 of the PO-CHH cDNA. Marker lengths are shown in kb. (**b**) Schematic representation of the CHH genes showing restriction sites, sizes of exons and introns, and the choice of PCR primers used for cloning. The *Spe*I site (in italics) in the second intron is not present in all genes.

cDNAs regardless of the length of the 3′-UTR resulted in multiple hybridizing genomic DNA fragments of approx. 3.5 kb. Consequently, the total intronic sequences covered by this fragment are estimated to be around 2.5 kb. Additional PCR analysis of genomic DNA with 3′-UTR-specific primer pairs confirmed a total size of about 4 kb for the *Carcinus* CHH genes

Table 4 Positions, lengths and flanking sequences of the introns found in this study

		Sequence			
Intron	Position on cDNA	Exon	Intron	Exon	Length
I	89	AAAACT	GTaagt...ttttactccAG	ATTCCC	505–1000
II	391	CTGCAG	GTgggt...gttgtttgcAG	GAATAA	700–850
III	515	ATTAGG	GTaatg...taccatttcAG	ATCAAA	376 or 500

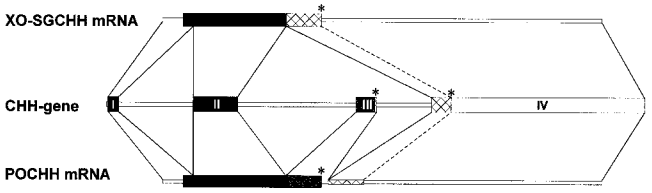

Figure 8 Putative splicing events leading to PO- and X-organ-type CHH mRNAs

The common coding region for the signal peptide, CPRP and the N-terminus of the mature peptide (exons I and II) is represented by black boxes. The coding regions for the C-termini of the mature peptides are shown as dark grey (exon III) or cross-hatched (part of exon IV) boxes, respectively. The locations of stop codons is indicated by asterisks.

and the absence of additional exons in the 3′-UTR (results not shown). A representative gene structure and sequence was compiled from three overlapping PCR amplicons (Figure 7b; GenBank accession numbers AF288680–AF288682), showing that the coding part is divided into four exons separated by three introns (I–III; 505, 796 and 376 bp in length, respectively; Table 4).

The first exon codes for the first five amino acids of the signal peptide. The remaining signal peptide residues, the entire CPRP and the first 40 amino acids of the mature CHH peptides are encoded by exon II. Exons III and IV code for the C-terminal remaining sequences of the PO-CHH and SG-CHH, respectively. The long form of the 3′-UTR as described earlier for the SG-CHH precursor was found on exon IV. So far, we have found no evidence for genes bearing the information for a shorter 3′-UTR, nor did we observe PCR signals indicating the existence of genes lacking exon III, i.e. genes coding exclusively for a SG-CHH. Thus we conclude that the two CHH isoforms described here are products of differential splicing (Figure 8). With the exception of intron III, for which only two size variants have been observed, the introns were highly variant in size, mostly due to length differences of two–five base repeats, whereas the base composition itself was rather conserved. All introns (Figure 7b and Table 4) displayed canonical GT–AG boundaries and were flanked by consensus matching exonic acceptor and donor sequences. In all cases of introns I and II, we observed at least four different forms, confirming the minimum number of four gene copies concluded from the restriction-digestion experiments. In general, the coding sequences derived from PCR on genomic DNA were identical to the multiple cDNA types revealed by RT-PCR. In particular, the PO-CHH-Q^{55}/N^{60}-encoding variant was also found without further modifications in the genomic DNA, but the majority of detected clones coded for a PO-CHH-E^{55}/D^{60} variant, although this was not detected in independent RP-HPLC-separated PO extracts.

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DISCUSSION

The intrinsic multipolar neurosecretory cells in the POs of *C. maenas*, immunoreactive to all tested antisera against SG-CHH, SG-CPRP and the C-terminal hendecapeptide of PO-CHH, match exactly those described histologically 40 years ago in this crab species in terms of number, shape and distribution, and resemble those in other crab species [36]. These cells comprise the only intrinsic cell type in the POs and the only hitherto known extra-eyestalk source of CHH-like molecules, excepting the recent finding of CHH in gut endocrine cells of *C. maenas* [11]. The cells are considered the origin of the novel PO-CHH, which is obviously released from the observed terminals at the surface of the POs, as confirmed by our release experiments. These cells are similar to anti-CHH immunopositive peripheral cells in comparable neurohaemal release sites, as described recently in *Daphnia magna*, *Artemia salina*, *Locusta migratoria*, *Homarus gammarus* and *H. americanus* [18,20,37,38], suggesting similar if not homologous cell types in different arthropod groups. Forms and distributions of varicosities and release terminals of these cells which occur over almost all the trunks and bars of the crab POs resemble those of previously described neurons containing cardioactive peptides, such as proctolin, crustacean cardioactive peptide, FMRFamide-related and allatostatin-related peptides, all of which originate in the thoracic ganglia (see [39,40], and H. Dircksen, unpublished work).

Among many CHH-like peptides described from SGs of several decapod and isopod crustaceans, PO-CHH is a novel CHH-like peptide from the crab peripheral nervous system not only in terms of its primary structure but also with regard to its apparent lack of hyperglycaemic action. PO-CHH also did not significantly inhibit ecdysteroidogenesis of the crab Y-organ, a typical effect of the moult-inhibiting hormone (MIH) and to a lesser extent of the SG-CHH [32]. However, since PO-CHH enhanced cGMP production in Y-organs significantly (though to a lesser extent than SG-CHH), a possibility exists that PO-CHH is recognized by the SG-CHH receptor on this tissue, but that signal-transduction mechanisms are ameliorated to such an extent that activation of a cGMP-dependent protein kinase [33,41] may not result in a detectable biologically relevant response. Since PO-CHH produced a detectable but non-significant inhibition of Y-organ ecdysteroidogenesis and its most significant effects on Y-organ cGMP production obviously during winter time (Tables 2 and 3), more experiments are needed to explore the possibility of circannually different effects, if these exist and occur at corresponding haemolymph concentrations of PO-CHH, which are currently being investigated. In contrast to SG-CHH, PO-CHH had virtually no effect on heart-muscle cGMP synthesis. Thus the possible functions of PO-CHH still remain elusive. In view of the rather limited quantities of PO-CHH available for release (the PO-CHH content of a PO is between one-fifth and one-tenth that of the SG-CHH content of a SG) and the architecture of the neurosecretory cells, it seems reasonable to suggest that hormone release from these cells may not be systemic, i.e. it seems possible that these cells have a sensory function and may act as local modulators. The position of these cells in the openings of the branchiocardiac veins into the pericardial cavity suggests that they may sense physicochemical changes in haemolymph composition.

The bioassay results were unexpected, because the structural similarities of PO-CHH and SG-CHH in *C. maenas* are striking at the level of the amino acid and nucleotide sequences, although the C-terminal moieties (sequence domains following amino acid 40) of the molecules are largely different, which therefore may imply the existence of distinct (C-terminal) receptor-recognition sites for SG-CHH responsible for its known diversified bioactivities. In recent years, a multitude of CHH-like peptides have been identified, and it is extremely difficult to assign single physiological roles to these peptides. In the present case we have a unique example where a peptide closely related to one which has well established functions (SG-CHH) does not appear to fulfil such a role. Thus we propose that PO-CHH has other unknown functions and, furthermore, we propose a hypothesis in which we consider that variation in the C-terminus of all CHH neuropeptides may define their functions, although their similarity in the N-terminal region may result in receptor occupancy (e.g. on Y-organs). Full biological activity and, in consequence, uncompromised signal-transduction pathways cannot occur without a 'perfect fit' of ligand to receptor, hence selectivity can occur and physiological mechanisms can be integrated despite the presence of a plethora of similar, presumptive, neurohormonal peptides.

Our RT-PCR experiments have established the existence of a PO-specific CHH-like peptide that differs significantly from SG-CHH in the C-terminal sequence. Moreover, we have shown that the multitude of different CHHs in one animal can be partially explained by differential splicing events, a phenomenon which becomes even more complex by the provided evidence for the existence of multiple copies of CHH genes in the shore crab. When considering only the predominant hybridization signals obtained in the restriction-digest experiments and the major differences in intron length observed in the genomic clones, we must postulate the existence of at least four CHH genes. However, when judging the number of slightly different cDNAs found in the present study, the actual copy number is most likely somewhat higher. In addition, we can assume the existence of silent or non-functional genes, since we have found genomic sequences (not shown here) that were not observed at the cDNA or peptide levels.

The existence of multiple gene copies seems to be a common feature of the CHH family. Similar findings have previously been described for the shrimp *M. ensis*, in which at least eight different CHH genes have been found [9,10,42]. Detailed analyses of the mandibular organ-inhibiting hormone and MIH genes of *Cancer pagurus* led the authors to postulate up to 10 gene copies per genome [43,44]. Six different CHH-like cDNAs have been identified in the shrimp *P. monodon* [12,45]. In *Penaeus japonicus*, five similar CHH-like SG peptides have been characterized [46,47]. For the crayfish *O. limosus* and *Procambarus clarkii*, and for the lobster *H. americanus*, two CHH-like SG-peptides (plus one or two stereoisomers in crayfish and lobster, respectively) and two different cDNAs (see [1,48]) have been demonstrated. Furthermore, several structural characteristics of the genes coding for peptides of the crustacean CHH/insect ITP family seem to be remarkably conserved within the decapod crustacean group. All analyses of genes of the CHH family performed so far have revealed a similar organization of exons and introns. In most cases a short first exon coding for a stretch of the signal peptide precedes a second exon coding for the remaining signal-peptide residues (with the exception of a cDNA encoding the total signal peptide in a *P. monodon* gene [12]) plus the entire CPRP and the 40 N-terminal amino acids of CHH-like peptide(s) and finally at least one copy of the third exon coding for the C-terminal residues of the mature CHH-like peptide. Interestingly, a similar arrangement of exons and introns has recently been described for a MIH gene in the crab *Charybdis feriatus* [49] and for the mandibular organ-inhibiting hormone and MIH genes in the crab *C. pagurus* [44]. In contrast, in the crab *C. maenas*, two different copies of the last exon are present on the CHH genes. Tissue-specific splicing would either generate a PO-specific CHH

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(no exons spliced) or a SG-specific CHH (exon III excised; Figure 8). Similar duplications or even multiplications of exon III occur in other arthropod species. In the European lobster *H. gammarus*, we have found different CHH cDNAs in the MTs and thoracic segmental nerve roots, and the longer isoform in the roots contained the coding region of the C-terminal moiety of a SG-CHH-peptide isoform in the 3′-UTR [38]. Likewise, a sequence stretch in the second 'intron' of the giant freshwater shrimp *Macrobrachium lanchesteri* (GenBank accession number AF088854) would code for a deduced amino acid sequence displaying all invariant characteristics of a truncated peptide fragment (sequence starting at amino acid 41) that is the motif X(N/D)C(F/Y)XXXXFXXCXXXL. The organization of insect ITP genes is probably very similar. For the locusts *Schistocerca gregaria* and *L. migratoria*, two ITP cDNA isoforms expressed in different tissues have been described [14,50]. The longer isoform contains the coding sequence for a C-terminal peptide fragment (starting at amino acid 41) of the shorter ITP isoform in its 3′-UTR, similar to the situation in the crab. From a comparative point of view, the short form of ITPs appears similar to the SG-CHH, and the long form of ITP, which does not stimulate ion transport [14], may resemble a PO-CHH. Most recently, the *Drosophila* genome project revealed a gene for a putative *Drosophila* ITP (GenBank accession number AE003463) that contains three predicted exons for different C-termini (from amino acid 41). In contrast, none of the CHH genes of the shrimps *M. ensis* [9,10] and *P. monodon* [12] bears more than three exons, and the data available for CHH cDNAs of *P. japonicus* and *P. monodon* thus far do not indicate the existence of any PO-CHH-like peptide in these species, which belong to a phylogenetically modern crustacean group distant from other decapods. On the other hand, the SG-CHH isoforms of all crustaceans show a high degree of homology in their C-termini encoded by a third or a fourth exon. However, the C-terminus of the crab PO-CHH, encoded by exon III of the CHH gene, appears considerably different. To date, there is no similar peptide known for any other crustacean species except for lobsters [38].

Only three out of about 15 conceptual translations of cDNA and genomic sequences analysed in the present study resulted in amino acid sequences that were completely identical to the PO-CHH peptide sequence described above, i.e. coding for the Q^{55} and N^{60} residues instead of E^{55} and D^{60}. However, our RP-HPLC/dot-immunobinding assay results show that this peptide is the exclusive CHH-like isoform present in POs. Even if the observed heterogeneity at the mRNA level is considered to be caused by allelic variation between individuals and/or gene copies, or by PCR artifacts, the phenomenon of expression of a major translated peptide not being exclusively accompanied by an entirely corresponding major mRNA isoform appears unusual. At present, neither mechanisms known for metazoan RNA-editing processes nor known post-translational protein modifications, which differ from presumptive amidations of intrachain acidic amino acid residues as in our case, can fully account for the observed differences. Moreover, the existence and possible functional significance of the predominant shorter 3′-UTRs observed in PO-CHH cDNAs or the mechanisms underlying the tissue-specific usage of the variant splice sites merit further study. As a possible reason for the former, an initial mispriming of the oligo-dT primer to particular A-rich sequences during reverse transcription cannot be ruled out, which might gain support from the fact that the Northern blot showed only one transcript of about 2 kb. Since this length of transcript does not correspond to any of the shorter PO-CHH cDNAs observed (Figure 4), we have to assume that the majority of transcripts initially have a longer 3′-UTR, as has been found first in the SG-CHH cDNA and confirmed here by our genomic sequencing results. However, together with the complete absence observed in PO-CHH cDNAs of a 110 bp-long intercalating 3′-UTR CT repeat, which exists in the SG-CHH cDNA as well as in the CHH gene itself, this conclusion implies that some complex hitherto unknown mRNA-processing events occur in crabs.

For the past 40 years it has been tacitly assumed that hyperglycaemic hormones are involved in energy metabolism. This assumption has been challenged by numerous workers, and their results suggest that CHHs are pleiotropic hormones and possibly affect every aspect of crustacean physiology in the similar way that insulin affects almost every aspect of vertebrate physiology. Our current finding is noteworthy in that we have found a translated product that does not have established CHH-like activities. Moreover, we have shown that there are several gene products that may undergo possibly novel post-transcriptional or post-translational modifications. We are just beginning to understand the complex functions of CHHs but, in our opinion, functional genomics must identify products that are translated. In the present study, we have evidence to suppose that results from PCR-based studies should be treated with some caution. Only when we have identified the proteomic profiles of a multigene family such as the CHH family in relation to the respective mRNAs can we proceed to rigorously elucidate the functions of these peptides.

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