Molecular Cloning of Three Distinct cDNAs, Each Encoding a Different Adipokinetic Hormone Precursor, of the Migratory Locust, Locusta migratoria

DIFERENTIAL EXPRESSION OF THE DISTINCT ADIPOKINETIC HORMONE PRECURSOR GENES DURING FLIGHT ACTIVITY* (Received for publication, May 12, 1995, and in revised form, July 5, 1995)

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Three distinct cDNAs encoding the preproadipokinetic hormones I, II, and III (prepro-AKH I, II, and III), respectively, of Locusta migratoria have been isolated and sequenced. The three L. migratoria AKH precursors have an overall architecture similar to that of other precursors of the AKH/red pigment-concentrating hormone (RPCH) family identified so far. The AKH I and II precursors of L. migratoria are highly homologous to the Schistocerca gregaria and Schistocerca nitans AKH precursors. Although the L. migratoria AKH III precursor appears to be the least homologous to the Manduca sexta, Drosophila melanogaster, and Carcinus maenas AKH/RPCH precursors, we favor the opinion that the L. migratoria AKH III precursor is evolutionary more related to the M. sexta, D. melanogaster, and C. maenas AKH/RPCH precursors than to the AKH I and II precursors of S. gregaria, S. nitans, or L. migratoria. In situ hybridization showed signals for the different AKH mRNAs to be co-localized in cell bodies of the glandular lobes of the corpora cardiaca. Northern blot analysis revealed the presence of single mRNA species encoding the AKH I precursor (~570 bases), AKH II precursor (~600 bases), and AKH III precursor (~670 bases), respectively. Interestingly, flight activity increased steady-state levels of the AKH I and II mRNAs (~2.0 times each) and the AKH III mRNA (~4.2 times) in the corpora cardiaca.

Three peptide hormones with hyperlipemic activity, the adipokinetic hormones I, II and III (AKH I, II and III; see Table I) (1–3), are synthesized by the glandular neurosecretory cells of the corpora cardiaca (CC) of the migratory locust, Locusta migratoria. These peptides are members of a large family of structurally related but functionally diverse peptides (the AKH/RPCH family) (4). In the adult locust, the AKHs I and II are released into the hemolymph during flight and are involved in the mobilization of lipid and carbohydrate from the fat body to serve as energy substrates for the flight muscles (4–7). Data on the release and functioning of AKH III are lacking so far. Isolation and characterization of CC peptides revealed that two other locust species, Schistocerca gregaria and Schistocerca nitans, each contain two AKHs that are mutually identical (1, 8), whereas Manduca sexta and Drosophila melanogaster each contain only one AKH (9, 10) (see Table I).

Molecular biological studies have resulted in the characterization of the AKH/RPCH precursors (a signal peptide, AKH/RPCH, a Gly-(Lys/Arg)-Arg sequence, and an AKH/RPCH-associated peptide (AAP/RAP), in this order) of S. gregaria, S. nitans, M. sexta, D. melanogaster, and Carcinus maenas (11–17).

The biosynthesis of the AKHs in S. gregaria has been elucidated in detail by O’Shea and co-workers (13, 18–21). The signal peptide is co-translationally removed from prepro-AKH, generating pro-AKH. Next, proteolytic processing, which is preceded by dimORIZATION of two pro-AKHSs (I/I, I/I, or I/I) via their single COOH-terminal Cys residues, gives rise to two AKHs (I and/or II) and one homo- or heterodimeric peptide consisting of two AAPSs (I/I, I/I, or I/I), a so-called AKH precursor-related peptide, as end products. The biosynthesis of AKH I and II of L. migratoria proceeds via the same pathway (3).

For the migratory locust, we now present three cDNA sequences, each encoding a different AKH precursor. The present data show that the AKH I and II precursors of L. migratoria are highly homologous to their S. gregaria and S. nitans counterparts. The AKH III precursor of L. migratoria appears to be more homologous to the L. migratoria, S. gregaria, and S. nitans AKH I and II precursors than to the M. sexta, D. melanogaster, and C. maenas AKH/RPCH precursors. In addition, we show that flight activity differentially increases the level of each prepro-AKH mRNA.

MATERIALS AND METHODS

Preparation of mRNA and Polymerase Chain Reaction—Total RNA was extracted from CC of male L. migratoria by the method of Chirgwin et al. (22). Poly(A)-rich RNA was prepared using Dynabeads-oligo dT25 (Dynal A.S.). Partial pre-AKH cDNAs were amplified from the RNA based on the principle of 3’ RACE (23). Using a DNA Thermal Cycler (Perkin-Elmer), each polymerase chain reaction (PCR) amplification was performed in a 100-μl reaction mixture containing 10 μl of 10 × PCR buffer (HT Biotechnology Ltd.), 200 μM dNTPs (Pharmacia), primers and templates as indicated (Table II), and 1 unit of SuperTag DNA polymerase (HT Biotechnology Ltd.). The mixture was overlaid with 70 μl of light mineral oil (Sigma). Oligodeoxynucleotide Primers—Oligodeoxynucleotide primers were obtained from Pharmacia: oligo(dT)15 adaptor primer, 5’-GACTCGAGTCGACATCGATT-3’; adaptor I primer,

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1 The abbreviations used are: AKH, adipokinetic hormone; AAP, AKH-associated peptide; bp, base pair(s); CC, corpora cardiaca; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RAP, RPCH-associated peptide; RPCH, red pigment-concentrating hormone.
5' CGCGGTACCAAATTAACCCTCACTAAAGGG-3'; and T7-PCR primer, 5' CGCGGATCCCA(A/G)CT(G/A/T/C)TGG-3'.

To generate AKH-C primer, a degenerate AKH-B primer, and T7-PCR primer, the T7-PCR primers were designed based on the amino acid sequences of AKHs I, II, and III (amino acids 1–9), respectively; the degenerate AKH-A primer was designed based on the amino acid sequences of the AKHs I, II, and III (amino acids 1–7).

Degenerate primers were used in a nested PCR reaction to amplify fragments of cDNA pool. The reaction mixture for the first PCR (PCR I) contained 0.5 μM degenerate AKH-A primer and 0.5 μM degenerate AKH-B primer and 0.25 μM degenerate AKH-C primer. The reaction mixture for the second PCR (PCR II) contained 0.5 μM degenerate AKH-B primer and 0.5 μM degenerate AKH-C primer and 0.5 μM adaptor II primer.

The reaction mixture for the third PCR (PCR III) contained 0.5 μM degenerate AKH-B primer and 0.5 μM adaptor II primer and 0.25 μM adaptor III primer. The reaction mixture for the fourth PCR (PCR IV) contained 0.5 μM degenerate AKH-C primer and 0.5 μM adaptor II primer and 0.25 μM adaptor III primer.

Table II

| Name | Sequence |
|------|----------|
| Lom AKH I | <Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH<sub>2</sub> |
| Scg AKH I | <Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH<sub>2</sub> |
| Scg AKH II | <Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH<sub>2</sub> |
| Snc AKH II | <Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH<sub>2</sub> |
| Mas AKH | <Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH<sub>2</sub> |
| Drm AKH | <Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH<sub>2</sub> |

Oligonucleotide primers and cycling parameters for 3' RACE of partial pro-AKH cDNAs of L. migratoria in particular PCR experiments:

| PCR | Primer 1 | Primer 2 | Template | Denaturation | Annealing | Polymerization | Cycles<sup>a</sup> |
|-----|----------|----------|----------|-------------|-----------|---------------|-----------------|
| I   | D-AKH-A  | ADA I    | cDNA     | 94          | 45        | 48            | 60              | 72              | 60              | 15              |
| II  | D-AKH-A  | ADA I    | PCR I    | 94          | 45        | 56            | 30              | 72              | 60              | 20              |
| III | D-AKH-B  | ADA I    | cDNA     | 94          | 45        | 48            | 60              | 72              | 60              | 15              |
| IV  | D-AKH-C  | ADA I    | PCR III  | 94          | 45        | 60            | 30              | 72              | 60              | 20              |
| V   | D-AKH-C  | ADA I    | PCR IV   | 94          | 45        | 53            | 30              | 72              | 60              | 5               |

<sup>a</sup>The final cycle of each PCR amplification included polymerization for 7 min for complete strand extension.
25-cmcassettes at room temperature. After exposure, phosphorscreens were scanned on a PhosphorImager SI (Molecular Dynamics), and phosphorimages were analyzed with ImageQuant version 4.1 software (Molecular Dynamics).

RESULTS

Primary Structure of the Prepro-AKH I, II, and III mRNAs—Partial pro-AKH cDNAs were amplified from L. migratoria CCmRNAbased on the principle of 3'-RACE described by Frohman (23). To this end, we used degenerate oligodeoxynucleotide primers based on the amino acid sequences of the AKHs I, II and III (see Table II). Two distinct bands of PCR products (PCR II) of approximately 380 and 300 bp and one band of PCR products (PCR V) of approximately 450 bp were detected in agarose gel electrophoresis. The putative pro-AKH I and II cDNAs were identified by sequence analysis of several clones derived from both the 380- and the 300-bp bands, whereas the putative pro-AKH III cDNA was identified by sequence analysis of several clones derived from the 450-bp band.

Subsequently, the three different pro-AKH 3'-RACE products were used to screen a L. migratoria CC-specific cDNA library. The nucleotide sequences of the longest AKH I, II, and III cDNAs and their deduced amino acid sequences are shown in Figs. 1–3. The AKH I cDNA consists of 363 bp, including an open reading frame of 189 nucleotides encoding the L. migratoria AKH I precursor. The AKH II cDNA consists of 367 bp, including an open reading frame of 183 nucleotides encoding the L. migratoria AKH II precursor. The AKH III cDNA consists of 438 bp, including an open reading frame of 231 nucleotides encoding the L. migratoria AKH III precursor.

Expression of the Prepro-AKH I, II, and III Transcripts—The sites of expression of the three AKH mRNAs in the L. migratoria CC were studied using in situ hybridization. Cell bodies of L. migratoria AKH I, II, and III prohormones contain Gly-Lys-Arg (for pro-AKH I and III) or Gly-Arg-Arg (for pro-AKH II) sequences, of which the (Lys/Arg)-Arg pairs may serve as sites for prohormone convertase processing and of which the Gly is considered as the donor for COOH-terminal amidation of the AKHs. If these sites are recognized, AKH I (amino acids 1–10) and AAP I (amino acids 14–41) are generated from the AKH I prohormone. In a similar way, AKH II (amino acids 1–8) and AAP II (amino acids 12–39) are generated from the AKH II prohormone, and AKH III (amino acid 1–8) and AAP III (amino acid 12–55) are generated from the AKH III prohormone.

Expression of the Prepro-AKH I, II, and III Transcripts—The sites of expression of the three AKH mRNAs in the L. migratoria CC were studied using in situ hybridization. Cell bodies...
Inc. (Life Technologies, 0.1% SDS at 65°C for 2 15 min for standardization of the amount of RNA loaded in each lane.

![In situ hybridization of corpora cardica of L. migratoria. Alternate transverse sections through the CC containing cell bodies that show in situ hybridization signals for the pro-AKH I mRNA (I), for the pro-AKH II mRNA (II), and for the pro-AKH III mRNA (III).](image)

**Fig. 4. In situ hybridization of corpora cardica of L. migratoria. Alternate transverse sections through the CC containing cell bodies that show in situ hybridization signals for the pro-AKH I mRNA (I), for the pro-AKH II mRNA (II), and for the pro-AKH III mRNA (III).**

![Northern blot analysis of L. migratoria AKH precursor mRNAs. A, hybridization of RNA isolated from CC of locusts that had flown for 1 h (lanes indicated with F) as well as from CC of resting locusts (lanes indicated with R) with pro-AKH I cDNA probe (I), with pro-AKH II cDNA probe (II), and with pro-AKH III cDNA probe (III) after washing with 1× SSC, 0.1% SDS at 65°C for 2 × 15 min. Indicated are the 0.16–1.77-kb RNA size markers (Life Technologies, Inc.). B, after stripping off the AKH cDNA probes, the membranes were hybridized with an 18 S RNA cDNA probe and washed with 1× SSC, 0.1% SDS at 65°C for 2 × 15 min for standardization of the amount of RNA loaded in each lane.](image)

**Fig. 5. Northern blot analysis of L. migratoria AKH precursor mRNAs. A, hybridization of RNA isolated from CC of locusts that had flown for 1 h (lanes indicated with F) as well as from CC of resting locusts (lanes indicated with R) with pro-AKH I cDNA probe (I), with pro-AKH II cDNA probe (II), and with pro-AKH III cDNA probe (III) after washing with 1× SSC, 0.1% SDS at 65°C for 2 × 15 min. Indicated are the 0.16–1.77-kb RNA size markers (Life Technologies, Inc.). B, after stripping off the AKH cDNA probes, the membranes were hybridized with an 18 S RNA cDNA probe and washed with 1× SSC, 0.1% SDS at 65°C for 2 × 15 min for standardization of the amount of RNA loaded in each lane.**

showing mainly co-localized signals for all three AKH mRNAs were present in the CC (Fig. 4). No hybridization signals were found using the sense RNA probes.

In order to examine the full-length AKH mRNAs as well as to study the effect of flight activity on the AKH gene expression, RNA was extracted from CC of locusts that had flown for 1 h as well as from CC of resting locusts. RNA blot analysis (Fig. 5) revealed that the mRNA encoding the AKH I precursor clearly is the most predominant AKH transcript expressed in the CC. In addition, flight activity caused the steady-state levels of the AKH I and AKH II transcripts and the AKH III transcripts to increase approximately 2.0 and 4.2 times, respectively. Northern blot analysis also showed that the cDNAs encoding the AKH I, II, and III precursors represent transcripts of −570, −600, and −670 bases, respectively.

**DISCUSSION**

Organization of the AKH I, II, and III Preprohormones—We have cloned and characterized three different abundant cDNAs, which are co-expressed in cells of the glandular lobes of the corpora cardica of L. migratoria. The proteins encoded by the cDNAs are organized as preprohormones. After co-translational removal of their 22-amino-acid signal peptides, the resulting prohormones are likely to be cleaved to generate two bioactive peptides. Isolation and characterization of L. migratoria CC peptides (3, 29) revealed that, indeed, the glycine residue in combination with the dibasic residues following the AKH sequence (residues 11–13 for the AKH I prohormone, and residues 9–11 for the AKH II and III prohormones; Figs. 1–3) are used as the actual signals for COOH-terminal amidation of AKH and prohormone convertase processing.

Further processing of the three different L. migratoria AAPs seems to be very unlikely, because at least unprocessed L. migratoria AAPs I and II have been isolated in the form of homo- or heterodimers, linked via their COOH-terminal Cys residues (29). In addition, also from S. gregaria and S. nitans, unprocessed AAPs can be isolated in the form of homo- or heterodimers. In both Schistocerca species this dimerization also has to precede the prohormone processing at the Gly-Lys-Arg or Gly-Arg-Arg sequences (7).

The presence of multiple bioactive peptides within single precursors is commonly observed (30). A consequence of such prohormone structures is that multiple companion peptides may coordinately be synthesized and released. If individual peptides within prohormones control different though related physiological and/or behavioral processes, this mode of synthesis and release may coordinate the component elements of a complex physiological and/or behavioral repertoire (31). This situation is even more complex for the peptides derived from the AKH I and II prohormones; dimerization of two pro-AKHS (I/I, I/II, or II/II) followed by proteolytic processing may give rise to different “bouquets” of AKHs (I and/or II) in combination with homo- or heterodimeric peptides consisting of two AAPs (I/I, I/II, or II/II). Data on the possible formation of intra- or intermolecular disulfide bridges of pro-AKH III are lacking so far.

Comparison of the AKH I, II, and III Preprohormones with Other AKH/RPCH Preprohormones—The amino acid sequences of the three L. migratoria AKH precursors were compared with the amino acid sequences of the AKH/RPCH precursors of S. gregaria (13–14), S. nitans (11), M. sexta (12), D. melanogaster (15), and C. maenas (17) (Fig. 6). As expected, the Locusta AKH I precursor revealed a high amino acid identity to the Schistocerca AKH I (89–92%) and II (59–63%) precursors, whereas the amino acid identity to the Manduca, Drosophila, and Carcinus AKH/RPCH precursors (∼30%) was much lower. In addition, the Locusta AKH II precursor also showed a high amino acid identity to the Schistocerca AKH I (57%) and II (80–82%) precursors. Again, the amino acid identity to the Manduca, Drosophila, and Carcinus AKH/RPCH precursors (∼30%) was lower. Nucleotide identity was also very high (i.e. 86% for the Locusta AKH I precursor with the Schistocerca...
AKH precursors and 84–86% for the Locusta AKH II precursor with the Schistocerca AKH II precursors). Comparison of the Locusta AKH III precursor with the AKH I and II precursors of L. migratoria, S. gregaria, and S. nitans showed relatively low percentages of amino acid and nucleotide identity. However, the L. migratoria AKH III precursor is different with respect to the AAP region; instead of 28 amino acids (the length of the AAPs I and II in Locusta and Schistocerca species), the AAP III is 44 amino acids in length. Interestingly, the M. sexta (Mas AKH), and D. melanogaster (Drm AKH), and the RPCH precursor of C. maenas (Cam RPCH).

**Fig. 6. Comparison of AKH/RPCH precursors.** A, the single-letter codes are used to designate amino acids. The three domains of signal sequence, AKH/RPCH, and AAP/RAP are set apart. Amino acids that represent the site for enzymic precursor cleavage and carboxyl-terminal amidation are joined to the AKH/RPCH sequence. Gaps (indicated by hyphens) were introduced optionally to achieve maximum similarity as well as taking into account conservative amino acid substitutions. B, UPGMA tree for the AKH precursors of L. migratoria (indicated by Lom AKH I, Lom AKH II, and Lom AKH III), S. gregaria (Scg AKH I and Scg AKH II), S. nitans (Scn AKH I and Scn AKH II), M. sexta (Mas AKH), and D. melanogaster (Drm AKH), and the RPCH precursor of C. maenas (Cam RPCH).

**Expression of the AKH I, II, and III Preprohormone Transcripts**—The results of in situ hybridization experiments (Fig. 4) showed that the signals for the three AKH-preprohormone mRNAs are co-localized in neurosecretory cells of the glandular lobes of the L. migratoria CC, which further extends earlier immunocytochemical observations using antisera specific for the AKHs I and II (32).

The results of the Northern blot analysis revealed that the prepro-AKH I, II, and III cDNAs very likely are full-length, assuming an average poly(A) tail of 200 nucleotides (Fig. 5). Interestingly, the ratio of steady-state AKH I, AKH II, and AKH III mRNA levels seems to be similar to the ratio of the AKH I, AKH II, and AKH III peptides (14:2:1) present in the CC of resting locusts (3, 33). Because each AKH may have a
different though related function, we reasoned that flight activity might induce a differential pattern of expression of the AKH genes in the CC. Indeed, a remarkable increase in the activity might induce a differential pattern of expression of the different though related function, we reasoned that flight activity (Fig. 5). Thus, the experiments suggest a stimulus-dependent differential pattern of expression of the AKH genes in one type of neurosecretory cell. The remarkable difference in flight-induced AKH III versus AKH I and II mRNA increase may shed new light on a possible role for AKH III during flight activity. In addition, these results are in accordance with the observed enhancement of the production of secretory granules by the trans-Golgi network in flight-stimulated adipokinetic cells of L. migratoria (34).

From the above experiments it may be concluded that the three different forms of AKH mRNA and as a result the three different forms of AKH precursors are co-expressed in the same cells of the corpora cardiaca of L. migratoria.

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