Differential Expression of SNMP-1 and SNMP-2 Proteins in Pheromone-Sensitive Hairs of Moths

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Abstract

In moths the detection of female-released sex pheromones involves hairlike structures on the male antenna. These long sensilla trichodea usually contain 2–3 chemosensory neurons accompanied by several supporting cells. Previous studies have shown that the pheromone-specific neurons are characterized by a “sensory neuron membrane protein” (SNMP) which is homologous to the CD36 family and localized in the dendrite membrane. By employing the SNMP-2 sequence from Manduca sexta we have isolated cDNAs that encode SNMP-2 proteins from Heliothis virescens (HvirSNMP-2) and Antheraea polyphemus (ApolSNMP-2). To elucidate the topographic and cell type–specific expression of these SNMP subtypes, 2-color in situ hybridization experiments were performed with tissue sections through the male antennae. For H. virescens, a specific probe for the pheromone receptor HR13 was used to identify pheromone-responsive neurons. It was found that HvirSNMP-1 and HR13 were coexpressed in the same cells; in contrast, HvirSNMP-2 was not expressed in HR13 cells but rather in cells that surrounded the HR13 neurons, apparently the supporting cells. A corresponding expression pattern was also found for ApolSNMP-1 and ApolSNMP-2 on the antenna of male A. polyphemus. Our results indicate that SNMP-1s and SNMP-2s are differentially expressed in cells of pheromone-sensitive sensilla and suggest distinct functions for the 2 SNMP subtypes in the olfactory system.

Key words: antenna, insect olfaction, moth, pheromone detection, sensilla trichodea, sensory neuron membrane protein

Introduction

Insects detect odor molecules through sensory neurons arrayed in olfactory sensory sensilla on the antenna. In moths, like the tobacco budworm Heliothis virescens and the silk moth Antheraea polyphemus, the detection of female-released pheromones involves particular classes of sensillar hairs on the male antenna (Kochansky et al. 1975; Almaas and Mustaparta 1991; Baker et al. 2004). These long sensilla trichodea typically contain 2–3 olfactory neurons surrounded by glia-like supporting cells (Steinbrecht and Gnatzy 1984; Meng et al. 1989). Neurons and supporting cells express a variety of specific proteins that give rise to the pheromone-sensitive phenotype. Supporting cells secrete pheromone-binding proteins (PBPs) and odorant-degrading enzymes (ODEs) into the lymph, which fills the sensillum and surrounds the dendrites of the sensory cells. PBPs are supposed to protect pheromone molecules from degradation and transport these hydrophobic compounds through the aqueous sensillum lymph toward specific receptors in the dendritic membrane of the sensory cells (Leal 2003; Vogt 2003; Grosse-Wilde et al. 2006; Grosse-Wilde et al. 2007). The ODEs appear to be involved in signal termination (Vogt and Riddiford 1981; Maida et al. 1995; Vogt 2003; Ishida and Leal 2005). The pheromone-responsive neurons gain the functional specificity through pheromone receptors, which are integral membrane proteins of chemosensory dendritic membranes (Krieger et al. 2004; Gohl and Krieger 2006). Moreover, sensory neurons seem to have an additional characteristic protein in the dendrite membrane, the “sensory neuron membrane protein” (SNMP). It was first identified in pheromone-sensitive neurons of A. polyphemus (ApolSNMP-1) (Rogers et al. 1997). Meanwhile, SNMP-1 homologs have been found in other moth species, including H. virescens (Rogers, Krieger, et al. 2001). SNMP-1 is specifically expressed in antennae and is localized in the dendrite membrane...
membrane of sensory cells (Rogers et al. 1997; Rogers, Steinbrecht, et al. 2001). SNMPs are homologous with members of the diverse CD36 receptor family, which in vertebrates mainly recognize proteinaceous ligands, cholesterol, and fatty acids (Febbraio and Silverstein 2007; Levy et al. 2007; Nassir et al. 2007). In addition, several CD36 homologs have been identified in insects, including proteins with functions in carotenoid transport (Voolstra et al. 2006; Wang et al. 2007). By homology to the CD36 family, SNMPs have been considered as potential elements involved in recognizing the lipophilic pheromone compounds (Rogers, Krieger, et al. 2001; Vogt 2003). A second SNMP subtype from *Manduca sexta* with a sequence identity of about 27% has been identified and designated as *MsexSNMP-2* (Rogers, Krieger, et al. 2001). Therefore, in this study, we have tried to identify additional SNMP subtypes from other moth species and to determine the topographic and cell-specific expression of SNMPs in the antenna.

**Materials and methods**

**Animals**

*Heliothis virescens* pupae were kindly provided by Bayer CropScience AG, Frankfurt, Germany. *Antheraea polyphemus* cocoons were obtained from Bill Oelke, Montague, Canada. Animals were sexed and allowed to develop into adults at 26 °C.

**Identification of SNMP-2 sequences**

To find SNMP homologs in *H. virescens*, we performed BLAST analyses (Altschul et al. 1990, 1997) of the nonpublic *Heliothis* genomic database using the coding region of *M. sexta* SNMP-2 (Rogers, Krieger, et al. 2001). A genomic sequence encoding an amino acid sequence highly similar to amino acid 1–410 of *M. sexta* SNMP-2 was identified. The expression of the gene in the antenna was verified by a reverse transcription–polymerase chain reaction (RT-PCR) including primers based on the genomic sequence and antennal cDNA. Polymerase chain reaction (PCR) conditions were as follows: 1 min 40 s at 94 °C, then 19 cycles with 94 °C for 1 min, 55 °C for 40 s, and 72 °C for 1 min, with a decrease of the annealing temperature by 0.5 °C per cycle. Subsequently, 19 further cycles at the condition of the last cycling step were performed followed by incubation for 7 min at 72 °C. PCR products of the size expected were cloned using the pGEM-T vector system (Promega, Mannheim, Germany) and verified by sequencing. Digoxigenin (DIG)-labeled probes for library screening were obtained by standard PCR using the DIG PCR labeling mixture (Roche, Mannheim, Germany), the cloned cDNA fragment as template and the corresponding primer pair. Labeled PCR products were gel purified by Gene clean and diluted in hybridization solution (30% formamide, 5x sodium chloride/sodium citrate [SSC], 0.1% lauroylsarcosine, 0.02% sodium dodecyl sulphate [SDS], 2% blocking reagent [Roche], and 100 µg/ml denatured herring sperm DNA).

**Screening of the antennal cDNA library**

DIG-labeled PCR fragments encoding SNMP-2–related sequences of *H. virescens* were used for the detection of corresponding full-length cDNAs in libraries made from the antennae of male *H. virescens* and *A. polyphemus*, respectively (Raming et al. 1989; Krieger et al. 1993). Screening of the cDNA library was performed as described (Krieger et al. 2002). Filters were hybridized to DIG-labeled probes at 37 °C. Posthybridization washes were twice for 5 min in 2x SSC, 0.1% SDS at room temperature, followed by 3 washes for 20 min each at 37 °C (*A. polyphemus*) or 60 °C (*H. virescens*) in 0.1x SSC, 0.1% SDS. Hybridized probes were detected using an anti-DIG alkaline phosphatase (AP)-conjugated antibody (Roche) and disodium 3-(4-methoxyxypirro[1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3,3,1,1,3,7]{decane}]-4-yl) phenyl phosphate (Applied Biosystems, Foster City, CA). cDNA inserts from positive phages were subcloned into the pBluescript II SK(+) vector and sequenced.

**Sequence analysis and comparisons**

Sequencing was performed on an ABI310 sequencing system using vector and cDNA-derived primers and the BigDye cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequence analyses and comparisons were made using the European Molecular Biology Laboratory (EMBL) Heidelberg unix sequence analysis resources (http://genius.embnet.dkfz-heidelberg.de). Based on a Clustal X (Thompson et al. 1994) alignment of the indicated protein sequences, a nonrooted neighbor-joining tree (Saitou and Nei 1987) was calculated with the MEGA program (Version 3.1) (Kumar et al. 2004). Pairwise differences were determined, ignoring missing characters resulting from alignment gapping. Tree branch lengths indicate percentage difference between sequences and are proportional. Bootstrap support of nodes is based on 1000 replicates.

**In situ hybridization**

Antennae of newly eclosed moths were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen on the object holder at −22 °C. Cryosections (12 µm) were mounted on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and air-dried at room temperature for at least 30 min. Two-color double in situ hybridizations with DIG- and biotin-labeled probes were performed as described previously (Krieger et al. 2002, 2004; Grosse-Wilde et al. 2007). Biotin-labeled probes were visualized using the tyramide-signal amplification kit (Perkin Elmer, Boston, MA), including an antibiotin streptavidin–HRP conjugate and fluorescein isothiocyanate-tyramides as substrate. DIG-labeled probes were detected by an anti-DIG AP-conjugated antibody in combination with 2-hydroxy-3-naphtoic
acid-2’-phenylanilide phosphate/Fast Red (Fluorescent detection Set; Roche). Sections were mounted using glycerol/phosphate buffered saline 3:1. Biotin-labeled or DIG-labeled antisense RNAs were generated from linearized recombinant Bluescript plasmids carrying the cDNA for HvirSNMP-1 (Rogers, Krieger, et al. 2001), HvirSNMP-2, ApolSNMP-1 (Rogers et al. 1997), ApolSNMP-2, HvirPBP1 (Krieger et al. 1993), or HR13 (Krieger et al. 2004). Transcription was performed following recommended protocols of the T3/T7 RNA transcription system (Roche).

Sections were analyzed on a Zeiss LSM 510 Meta laser scanning microscope. Images were arranged in PowerPoint (Microsoft). For uniform tone within a single figure, the brightness or contrast of single pictures was adjusted.

Data deposition
The sequences reported in this paper have been deposited in the EMBL database under the accession numbers AM905328 (HvirSNMP-2) and AM905329 (ApolSNMP-2).

Results
Identification of SNMP-2 sequences from H. virescens and A. polyphemus
Using the coding region of M. sexta SNMP-2 in BLAST analysis of a Heliothis genome database, we have identified a sequence encoding part of a homologous protein. Based on this sequence information, specific primers were synthesized, which allowed by RT-PCR to demonstrate that the gene was expressed in the antenna. Subsequently, a cDNA library from the antenna of male H. virescens was screened with a DIG-labeled probe, leading to the isolation of a cDNA clone of 1751 bp, which encodes a protein of 520 amino acids. The protein has 68% sequence identity to the M. sexta SNMP-2 and was referred to as H. virescens SNMP-2 (HvirSNMP-2). By low stringency screening of an antennal-derived cDNA library from A. polyphemus using a DIG-labeled probe of HvirSNMP-2, a homologous clone was isolated. Sequence analysis of the cDNA clone revealed an open reading frame for a protein (477 amino acid), which was highly related to HvirSNMP-2, but missed a start methionine and part of the N-terminus. Comparison of the HvirSNMP-2 and MsexSNMP-2 proteins with the A. polyphemus sequence revealed rather high identities of 67% and 72%, respectively. The newly identified SNMP-2–like protein from A. polyphemus was therefore named as ApolSNMP-2.

Among each other, the SNMP-2 types of the 3 moth species are much more similar (67–72%) than to the SNMP-1 proteins (25–29%). Similarly, the SNMP-1 proteins of different species have a rather high degree of sequence identity (66–90%). The relatedness of the 2 SNMP subtypes is well reflected in a nonrooted neighbor-joining tree (Figure 1).

Specific Proteins of Antennal Cells

2 SNMP subtypes from lepidopteran species each assemble in a common cluster.

Topographic and cell-specific expression of HvirSNMP-1 and HvirSNMP-2
To analyze the topographic expression of the 2 SNMP subtypes, double in situ hybridization experiments were
performed on cryosections through the antenna of *H. virescens* males. SNMP-specific probes were employed in combination with a probe specific for the pheromone receptor HR13, which responds to (Z)-11-hexadecenal, the main component of the female sex pheromone blend from *H. virescens* (Grosse-Wilde et al. 2007). Moreover, HR13 is expressed in a high number of cells (Gohl and Krieger 2006), which matches the number of long sensilla trichodea Type A, each containing a responsive neuron for (Z)-11-hexadecenal. (Almaas and Mustaparta 1990, 1991; Baker et al. 2004). To visualize cells which express HR13 and HvirSNMP-1, double in situ hybridizations were performed using a combination of a DIG-labeled HR13 probe and a biotin-labeled HvirSNMP-1 probe on the same section; DIG- and biotin-labeled antisense RNAs were visualized by different fluorescent colors. Probing longitudinal sections of the male antenna carrying long sensilla trichodea, the HR13 probe labeled single cells below this sensillum type (Figure 2), thus confirming previous observations (Krieger et al. 2004; Gohl and Krieger 2006). Compared with HR13 (Figure 2D), the HvirSNMP-1 probe generally labeled a higher number of cells per section (Figure 2C). An overlay of the HR13 and HvirSNMP-1 pictures (Figure 2B) revealed that all HR13-positive cells are also positive for HvirSNMP-1, indicating that the 2 proteins are expressed in the same cell. Interestingly, the HvirSNMP-1–labeled cells that did not express HR13 were never located in close proximity to the HR13 cells, although each long sensillum trichodeum usually contains 2 sensory neurons (Almaas and Mustaparta 1990; Baker et al. 2004). This finding suggests that the neighboring neurons of HR13 cells in the same sensillum do not express HvirSNMP-1 but rather may express another subtype. To scrutinize this notion, longitudinal sections of the male antenna were assessed with probes for HR13 and HvirSNMP-2. The results are documented in Figure 2E–K. It is immediately obvious that the probes did not label the same cells but rather different populations of cells. Moreover, all HvirSNMP-2–expressing cells were found in close association with HR13-positive cells. By optical sectioning of hybridized specimen at higher magnification (Figure 2F–K), it became clear that HR13-expressing cells were accompanied by HvirSNMP-2 cells. It is interesting to note that the green-labeled HvirSNMP-2 cells not only surround the red-labeled HR13 neuron but also a dark, nonlabeled spot (Figure 2G–J), which might be the space of a second neuron. Similar results were obtained in double in situ hybridization experiments with HvirSNMP-1 and HvirSNMP-2–specific probes; in accordance with the results seen with HR13, HvirSNMP-1–positive cells were found to be closely associated with HvirSNMP-2 cells (Figure 3).

### Expression pattern of SNMP-1 and SNMP-2 from *A. polyphemus*

The unexpected finding that each of the 2 SNMP subtypes from *H. virescens* is expressed in a distinct cell type, HvirSNMP-1 in sensory neurons and HvirSNMP-2 in supporting cells, prompted us to investigate whether a differential expression of the 2 SNMP subtypes also occurs in other moth species. Therefore, 2-color double in situ hybridization experiments were performed using specific probes for the 2 SNMPs from *A. polyphemus*. Tissue sections through side branches of male antennae from *Antheraea* were analyzed with DIG-labeled ApolSNMP-1 and biotin-labeled ApolSNMP-2 probes. The results documented in Figure 5 gave a picture quite similar to the results obtained for *H. virescens*. ApolSNMP-1 and ApolSNMP-2 were expressed in different cell populations (Figure 5A–C). ApolSNMP-1 neurons were found to be surrounded by ApolSNMP-2–expressing cells. Furthermore, as observed for *H. virescens*, only 1 ApolSNMP-1–positive cell was found to be associated with the ApolSNMP-2–expressing cells (Figure 5D–I).

### Discussion

In this study, we found for the moth species *H. virescens* and *A. polyphemus* that in only 1 of the 2 sensory neurons typically housed within a sensillum trichodeum (Meng et al. 1989; Almaas and Mustaparta 1990; Baker et al. 2004), SNMP-1 is expressed. This finding confirms the original observation on *A. polyphemus* (Rogers et al. 1997) but contradicts the results of electron microscopic studies, indicating that SNMP-1 may be present in the dendritic membrane of all neurons of an olfactory sensillum (Rogers, Steinbrecht, et al. 2001). These differences can currently not be reconciled and could either be due to very low levels of SNMP-1 mRNA in neurons neighboring SNMP-1–positive neurons or due to the fact that the SNMP-1 antibodies might cross-react with...
other proteins, such as yet unidentified SNMP isoforms. The latter view is supported by the identification of 2 neuron-specific SNMP subtypes from *M. sexta*, which share significant sequence similarity (Rogers, Krieger, et al. 2001).

The functional role of SNMP-1 in sensory neurons is still elusive, but due to its location in chemosensory dendrites of pheromone-sensitive neurons an immediate, as yet undefined role in pheromone detection has been suggested (Rogers et al. 1997). Due to the homology with members of the CD36 protein family, which interact with fatty acids (Fukuwatari et al. 1997; Gilbertson et al. 2005), lipids (Voolstra et al. 2006; van der Velde and Groen 2007), or...
lipid/protein complexes (Acton et al. 1996; Nieland et al. 2002, 2007), it has been speculated that SNMP may bind and transfer pheromone components or pheromone/PBP complexes (Rogers, Krieger, et al. 2001). In fact, it has been suggested that SNMP might act as a coreceptor, which brings an appropriate ligand to or in close proximity to the pheromone receptor (Vogt 2003). This notion has found strong support by the results of a recent study on Drosophila mutant flies lacking the expression of an SNMP-1 homologous protein in cells of trichoid sensilla. It was demonstrated that the SNMP is required for an electrophysiological responsiveness of OR67d neurons to the Drosophila pheromonal component cis-vaccenyl acetate (Benton et al. 2007). This observation was extended by experiments demonstrating that antennal neurons of Drosophila, which ectopically express the Heliothis pheromone receptor HR13, do respond to its cognate ligand, (Z)-11-hexadecenal, but only if the Drosophila SNMP-1 homolog is expressed (Benton et al. 2007). In view of this recent finding, our results that in H. virescens the antennal cells which express the pheromone receptor HR13 also express SNMP-1 (Figure 2) suggest that HvirSNMP-1 may contribute to the responsiveness of antennal neurons to pheromones. This aspect will be elucidated by coexpressing HvirSNMP-1 in our previously established heterologous system monitoring the response spectrum of candidate pheromone receptors (Grosse-Wilde et al. 2007).

Figure 3  In situ hybridization with HvirSNMP-1– and HvirSNMP-2–specific probes. A tissue section through a male antenna carrying long sensilla trichodea was hybridized with biotin-labeled HvirSNMP-1 and DIG-labeled HvirSNMP-2 antisense RNAs. Cells positive for HvirSNMP-1 and HvirSNMP-2 were visualized by green and red fluorescence, respectively. (A) Overlay of the transmitted-light channel with the red and green fluorescence channels. (B–D) Overlay (B) or separate presentation (C, D) of the green and red fluorescence channels. Scale bar: 20 μm. HvirSNMP-1 and HvirSNMP-2 are expressed in different subpopulations of cells located together under long trichoid hairs.

Figure 4  Coexpression of HvirSNMP-2 and HvirPBP1. DIG-labeled HvirSNMP-2 and biotin-labeled HvirPBP1 probes were used in two-color double in situ hybridization on a section through the male antenna. Positive cells were visualized by red (DIG) and green (biotin) fluorescence. The green (A) and red (B) fluorescence channels are shown separately and in an overlay with the transmitted-light channel (C). The HvirSNMP-2 and HvirPBP1 antisense RNA probes revealed a similar and overlapping labeling pattern, indicating expression of HvirSNMP-2 and HvirPBP1 in the same cells. Scale bars: 20 μm.
A major finding of this study was the identification of a novel SNMP subtype from Heliothis (HvirSNMP-2) and Antheraea (ApolSNMP-2), which was found to be expressed in the supporting cells of the sensilla (Figure 4). The expression of a novel SNMP-like protein in the supporting cells, of course, compromises the name “sensory neuron membrane protein”; in fact, the nomenclature should probably be reconsidered in the future. Here, we retained the name for the novel protein due to the clear sequence similarity with SNMP-1. There are as yet no findings concerning any functional implication of the SNMP-2 proteins in supporting cells. These glia-like cells not only generate the PBPs but also regulate the ionic and molecular composition of the sensillum lymph (Thurm and Küppers 1980; Steinbrecht et al. 1992) and thus ensure a proper function of the pheromone-sensitive unit. This task also includes a reiterative clearance of the sensillum lymph from lipophilic molecules, which, for example, result from degradation of pheromone components or are entering the hair from the air accidentally. The apparent relationship of SNMPs to proteins of the CD36 family, which mediate the selective uptake of fatty acids (Koonen et al. 2005; Levy et al. 2007; Nassir et al. 2007), carotenoids (Voolstra et al. 2006; Wang et al. 2007), and cholesterol esters (Acton et al. 1996; Ohgami et al. 2001), implies that SNMP-2 may play a role in the elimination of lipophilic components from the sensillum lymph. It may translocate the lipophilic compounds into the cell for further degradation and thus contribute to the clearance of the sensillum lymph, an essential prerequisite for the highly sensitive pheromone detection system. Although the specific function of the SNMP types yet needs to be determined, their differential expression in sensory neurons or in supporting cells suggests a multifunctional role. The observation that in Drosophila an SNMP-1 homolog is expressed in both neurons and supporting cells of trichoid sensilla (Benton et al. 2007) may point to a broader function of the dipteran SNMP protein;

**Figure 5**  Expression of ApolSNMP-1 and ApolSNMP-2 in the antenna of Antheraea polyphemus. A longitudinal section through a side branch of a male antenna was hybridized simultaneously with ApolSNMP-1 (DIG labeled) and ApolSNMP-2 (biotin labeled) antisense RNAs. Cells expressing ApolSNMP-1 are indicated by red fluorescence (C), whereas ApolSNMP-2–positive cells are labeled by green fluorescence (B). The overlay of the red and green fluorescence channels with the transmitted-light channel is shown in (A). (D–I) Higher magnification of the area boxed in (A); successive optical planes are shown. Scale bar: 20 μm.
on the other hand, the cell type-specific expression of distinct SNMP subtypes in the pheromone-responsive unit may indicate an evolutionary specialization of the moth olfactory system.

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