Expression analysis of putative vitellogenin and lipophorin receptors in honey bee (Apis mellifera L.) queens and workers

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ABSTRACT

Two members of the low density lipoprotein receptor (LDLR) family were identified as putative orthologs for a vitellogenin receptor (Amvgr) and a lipophorin receptor (Amlpr) in the Apis mellifera genome. Both receptor sequences have the structural motifs characteristic of LDLR family members and show a high degree of similarity with sequences of other insects. RT-PCR analysis of Amvgr and Amlpr expression detected the presence of both transcripts in different tissues of adult female (ovary, fat body, midgut, head and specifically hypopharyngeal gland), as well as in embryos. In the head RNA samples we found two variant forms of Amlpr: a full length one and a shorter one lacking 29 amino acids in the O-linked sugar domain. In ovaries the expression levels of the two honey bee LDLR members showed opposing trends: whereas Amvgr expression was upregulated as the ovaries became activated, Amlpr transcript levels gradually declined. In situ hybridization analysis performed on ovaries detected Amvgr mRNA exclusively in germ line cells and corroborated the qPCR results showing an increase in Amvgr gene expression concomitant with follicle growth.

1. Introduction

The insect yolk precursor protein, vitellogenin, is a lipoglycoprotein synthesized by the fat body and secreted into hemolymph from where it is sequestered by the growing oocytes via receptor-mediated endocytosis (Raikhel and Dhadiana, 1992). In the honey bee, native vitellogenin is a monomeric 180 kDa polypeptide (Wheeler and Kawooya, 1990) which, in egg-laying queens, accounts for over 50% of total hemolymph proteins (Hartfelder and Engels, 1998). Interestingly, in this social insect vitellogenin apparently has been co-opted into additional functions since it is also present in large amounts in the hemolymph of young worker bees, even though these are functionally sterile and do not lay eggs in the presence of the queen, and it has even been detected in drones (Trenczek et al., 1989; Piulachs et al., 2003; Guidugli et al., 2005a).

Additional functions for vitellogenin have also been suggested in other insects, such as the transport of carbohydrates, lipids, phosphates, vitamins, metals and hormones (see Chen et al., 1997; Sappington and Raikhel, 1998), but it is in the honey bee where vitellogenin has been co-opted into new roles integrating individual physiology with the social environment (Amdam et al., 2006). It has become a major regulator of the lifespan of individual worker bees, through its anti-immunosenescence properties as zinc transporter and antioxidant (Amdam et al., 2005; Seehues et al., 2006), and, in a double repressor circuitry with juvenile hormone, its downregulation drives a worker bee into the forager state (Amdam and Omholt, 2003; Guidugli et al., 2005b; Nelson et al., 2007). The emerging central role of vitellogenin in the life history of the honey bee, and possibly also in other highly eusocial bees (Hartfelder et al., 2006), now raises the question on how this protein may perform such proposed signaling functions, and a first candidate to investigate in this pathway should be the vitellogenin receptor.

Vitellogenin-specific receptors (VgRs) have been cloned and sequenced in several insects, such as Aedes aegypti (Sappington et al., 1996), Solenopsis invicta (Chen et al., 2004), Periplaneta americana (Tufail and Takeda, 2005), Blattella germanica (Ciudad et al., 2006) and Leucophaea maderae (Tufail and Takeda, 2007). This group also includes the related yolk protein receptor, yolkl ess, of Dro sophila melanogaster (Schonbaum et al., 1995). All these VgRs
are members of the superfamily of low density lipoprotein receptors (LDLR) and their expression appears to be restricted to the ovaries (Sappington et al., 1996; Schonbaum et al., 1995; Chen et al., 2004; Tufail and Takeda, 2005, 2007; Ciudad et al., 2006).

In accordance with the pleiotropic role of vitellogenin in Apis mellifera, immunodetection experiments now also evidenced an extraordinary occurrence of a putative VgR. An immunoreactive 205 kDa protein was detected not only in the ovaries of queens but also in the hypopharyngeal glands of workers (Amdam et al., 2003). These glands produce the major royal jelly proteins (Albert et al., 1999; Drapeau et al., 2007). Nurse bees feed royal jelly not only to the developing larvae, but also to the queen, allowing her to maintain the extraordinary high rates of egg production, thus establishing a social cycle for vitellogenin as a reproductive protein (Amdam et al., 2003).

Concomitant to the discovery of additional functions for insect vitellogenin, a dual role for lipophorin in reproduction has also been demonstrated in the moth and mosquito (Kawooya et al., 1988; Sun et al., 2000). In these insects, lipophorin is responsible for the shuttle of lipids from the fat body to the growing oocyte, and subsequently it is incorporated into the egg yolk as a storage protein. Like for vitellogenin, the incorporation of lipophorin also occurs via receptor-mediated endocytosis and the lipophorin receptor (LpR) also belongs to the LDLR superfamily.

The members of this superfamily share common structural characteristics and show a high degree of conservation in their functional domains. The structural elements of LDLRs are: (1) ligand-binding domain, (2) epidermal growth factor (EGF) precursor homology domain, (3) VVXX repeats, (4) transmembrane domain, and (5) cytoplasmic domain (for revision see Sappington and Raikhel, 1998; Rodenburg et al., 2006). Within the LDLR superfamily, VgRs and LpRs are intimately associated with oogenesis (Sappington et al., 1996; Schonbaum et al., 2000; Cho and Raikhel, 2001; Seo et al., 2003; Chen et al., 2004; Tufail and Takeda, 2005, 2007; Ciudad et al., 2006, 2007).

Considering the importance of vitellogenin and lipophorin in insect reproduction, we made use of the now available genomic information for the honey bee (The Honey Bee Genome Sequencing Consortium, 2006) to identify putative orthologs for a vitellogenin receptor and a lipophorin receptor, and investigated the expression of these genes in adult queens and workers and in different tissues, with emphasis on the reproductive process.

2. Material and methods

2.1. Bees and RNA extraction

A. mellifera workers and queens were collected from colonies of Africanized stocks maintained in the apiary of the Department of Genetics at the Faculty of Medicine in Ribeirão Preto, University of São Paulo, Brazil. Virgin queens obtained by standard queen rearing methods were either dissected immediately after emergence from brood cells or were introduced into queenless colony. They were collected as soon as they started to lay eggs. The ovaries from virgin and egg-laying queens were used for RNA extraction.

Newly emerged workers from a queenless colony were painted with blue dye to later be distinguished from ovipositing workers. They were collected in groups of 18 day-old workers. Active ovaries (stage II: initial follicle growth, where ovarioles contain mainly previtellogenic or early vitellogenic follicles; stage III: at least some of the ovarioles contain follicles in advanced stages of vitellogenesis) were observed in 15–20 and 20–30 day-old workers. After stage classification, the ovaries (pools of 10–20) were used for RNA extraction. In another experiment, RNA of inactive and active ovaries was obtained from queenless workers collected at the same age (18 days). RNA was also extracted from inactive ovaries of a group of 18 day-old queenright workers.

RNA was extracted from the head, fat body, hypopharyngeal glands, midgut and ovaries from adult workers and also from embryos (0–6 h) to test for tissue specificity of AmVgR and AmLpR gene expression. TRIzol reagent (Invitrogen) was used to isolate total RNA following manufacturer instructions. These RNA samples were used as templates for first-strand cDNA synthesis.

2.2. Cloning and partial sequencing of putative honey bee AmVgR and AmLpR genes

When the assembled honey bee genome (Amel version 4.0, http://hgsc.bcm.tmc.edu/projects/honeybee/) was searched for homologs to the yolkkine gene of D. melanogaster, the predicted honey bee gene (GB16571) was retrieved in a mutual best-hit analysis as the best candidate for a honey bee vitellogenin receptor. This predicted gene is subsequently referred to as Amvgr, indicating its putative function as vitellogenin receptor. Similarly, a putative honey bee lipophorin receptor (AmLpR) encoding gene was represented by CG11094-PA (GenBank accession no. XP_395858.3). BLASTP searches for closely related proteins predicted in the honey bee genome revealed an almost complete overlap in the five sequential best matches for the predicted AmVgR and AmLpR proteins, due to shared LDLR receptor domains (Supplementary Material, Table 1 and Figure 1).

To confirm that the in silico predicted genes Amvgr and AmLpR are expressed, we designed primers to amplify corresponding fragments by RT-PCR from ovary RNA. The PCR products separated by agarose gel electrophoresis were ligated into pGEM-T Easy (Promega) vector and cloned into chemically competent E. coli DH5α cells. Dideoxy sequencing was performed on an automatic sequencer (ABI Prism 310, Applied Biosystems) using BigDye Terminator v3.0 Cycle Sequencing Reaction (Applied Biosystems) and M13 forward and reverse primers.

2.3. Semi-quantitative expression analysis of putative honey bee Amvgr and AmLpR

The expression of Amvgr and AmLpR in various tissues and in embryos was evaluated by semi-quantitative RT-PCR. First-strand cDNA was synthesized by reverse transcription (SuperScript II, Invitrogen) using 1–2 μg of RNA. Aliquots of first-strand cDNAs were employed in PCR reactions using PCR Master Mix (Eppendorf). The primers were VgR1F: 5’-ACT CAT GTT TGT GCC AAC CGT-3’ and VgR2R: 5’-CTG ATC TGT ACC ACC CAA-3’ for Amvgr, and LpR5’-CAC TGG TCA ATC AGT TGA AG-3’ and LpRR 5’-CTA TAA CAT AAT ACT CTT AC-3’ for AmLpR. The Amvgr fragment was amplified by the following PCR protocol: 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C and a final extension step at 72 °C for 10 min. To amplify the fragment corresponding to the AmLpR, the thermal cycling program was: 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 60 s at 72 °C and a final extension step at 72 °C for 10 min. As loading control an A. mellifera actin gene (GenBank accession no. AB023025) was amplified following the protocol described by Bitondi et al. (2006).

The amplification products were analyzed by electrophoresis in 1% agarose gels containing ethidium bromide. In the case of AmLpR, the PCR products were Southern blotted. A cDNA probe was generated by PCR with the same primer pair and labeled with
fluorescein-11-dUTP using the module Gene Images Random Priming (GE Healthcare). The blots were hybridized with this gene-specific probe at 60 °C for 16 h before processing with Gene Images CPD Star (GE Healthcare) and autoradiography detection (Hyperfilm, GE Healthcare).

2.4. Quantitative expression analysis of putative honey bee Amvgr and Amlpr by real-time PCR

The levels of Amvgr and Amlpr mRNA in the ovaries of queens and in queenless and queenright workers with activated and non-activated ovaries was analyzed by a real-time quantitative RT-PCR (qPCR) protocol using a 7500 Real Time PCR System (Applied Biosystems). First-strand cDNA samples, previously analyzed by semi-quantitative RT-PCR, were diluted (1:5 or 1:10 v/v) in water, and 1 μl aliquots were used for qPCR. Amplifications were carried out in 20 μl reaction mixture containing 10 μl of SYBR® Green Master Mix 2x (Applied Biosystems), and 10 mM of Amvgr and Amlpr gene-specific forward and reverse primers (VgR forward: 5’-GTC GTT CAT TCA TAG GGC CAC GCC ACC TTC-3’; VgR reverse: 5’-CAT CGG ACC ACT AAC GCC ACC-3’), LpR forward: 5’-CGT CTT TCA ATA TCA TCA CTC-3’ and LpR reverse 5’-CGG ACA ACT AAC GAG-3’). The qPCR conditions were 50 °C for 2 min, and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. To check reproducibility, each SYBR® green assay was performed in triplicate. Relative quantities of Amvgr and Amlpr transcripts were calculated using the comparative Ct method (Applied Biosystems, User bulletin 2) with A. mellifera actin mRNA levels serving as internal control (Bitondi et al., 2006). The efficacy of this gene for normalization in qPCR studies on honey bee tissues and life cycle stages has been validated in our laboratory (Lourenço et al., 2008).

2.5. In situ hybridization

For in situ detection of transcripts of the putative vitellogenin receptor Amvgr, sense and antisense probes were synthesized using Amvgr specific primers with a T7 promoter sequence at the 5’-ends (VgR-Fow1: 5’-ATA TAC GAC TCA CTA TAG GGC CAA CTC ATG TTT GTG CCA ACC TG-3’; VgR-Rev1: 5’-ATA TAC GAC TCA CTA TAG GGC CAC CTT CTA GTA CCA TCC AA-3’) in combination with primers lacking the T7 sequence (VgR-Fow2: 5’-ACT CAT GTT TGT GCC AAC CTG-3’; VgR-Rev2: 5’-CCT TCG ATC TGT ACC ATC CAA-3’). These primers generated a product of 653 bp. The antisense and sense products were produced by the primer combination VgR-Fow2+ VgR-Rev1 and VgR-Fow1+ VgR-Rev2, respectively. Amplification conditions were: 94 °C for 2 min at 94 °C, 45 cycles of 94 °C for 40 s, 57 °C for 40 s, 72 °C for 40 s and a final extension step at 72 °C for 7 min.

Aliquots of the amplification products were checked on agarose gels, purified (Wizard® SV Gel and PCR Clean-Up System, Promega) and quantified spectrophotometrically. RNA probes were generated by in vitro transcription from the T7 promoter using the DIG RNA Labelling Kit (SP6/TT7) (Roche Applied Science). The transcription products were precipitated by addition of 1 μl ammonium acetate (10 M) and 20 μl isopropanol and washed in 70% ethanol. After evaporation of any ethanol residues they were resuspended in 50 μl hybridization buffer (50% formamide, 4× SSC, 1× Denhardt’s solution, 250 μg/ml yeast extract, 250 μg/ml salmon sperm DNA, 50 μg/ml heparin, 0.1% Tween-20, 5% dextrane sulfate) and stored at -20 C. Fixation of dissected tissue and the subsequent hybridization and detection reactions were performed following the protocol optimized by Osborne and Dearden (2005) for in situ hybridization studies on honey bees.

2.6. Phylogenetic analysis

Sequences used in the phylogenetic study were retrieved from the GenBank protein database. These included the insect VgRs of D. melanogaster (AA602017), Anopheles gambiae (AAE06264), A. aegypti (AAK15810), S. invicta (AAP92450), P. americana (BAC02725), L. maderae (BAE93218.1), B. germanica (CAJ19121), Tribolium castaneum (XP_968903.1) and Nasonia vitripennis (XP_001602954). The insect LpRs were from Galleria mellonella (ABF20542), Bombbyx mori (BAE71406.1), A. aegypti (AAQ16410 and AAK79254), D. melanogaster (NP_733119.1), A. gambiae (XP_307995), L. maderae (BAE00010), B. germanica (CAL7125), Locusta migratoria (CAAO3855), A. mellifera (XP_395858.3) and T. castaneum (XP_967944). Sequences from other ecodysozoa were the ovarian lipoprotein receptor (OLR) of the crustacean Penaeus semisulcatus (AAL79675.1), VgR of the tick Dermacentor variabilis (AAZ31260.3), and the yolk protein receptor RME-2 of the nematode Caenorhabditis elegans (AAD56241.1).

Vertebrates are represented by VgRs from Anguilla japonica (BAB64337.1, Conger myriaster (BAB64338, Oncorhynchus mykiss (CAD10640.1), Danio rerio (AAH47187), Xenopus laevis (AAH70552) and Gullus gallus (NP_990560); by the very low density lipoprotein receptor (VLDLR) of Oryctolagus cuniculus (BAA01874), Rattus norvegicus (NP_037287.1), Mus musculus (AAH13622.1) and Homo sapiens (NP_003374.3); by the LDLR of M. musculus (CAOA5759.1), R. norvegicus (NP_786938.1), Tetraodon nigroviridis (CA92585.1) and H. sapiens (NP_000518.1), and by the lipoprotein receptor-related protein (LRBB) of G. gallus (CAOA5729.1). A final addition were the megalins (=LRP2) of G. gallus (XP_422014.1), D. pseudoobscura (EAL32723) and A. mellifera (XP_393369).

Protein sequences were aligned using ClustalX (Thompson et al., 1997). Poorly aligned positions and divergent regions were eliminated by using Gblocks 0.91b (Castresana, 2000). The resulting alignment was analyzed by the PHYLm program (Guindon and Gascuel, 2003), based on the maximum-likelihood principle in the amino acid substitution model. Four substitution rate categories with a gamma shape parameter of 1.444 were used. Tree topologies were evaluated by 100 bootstrap replicates using PHYML.

3. Results

3.1. Putative vitellogenin and lipophorin receptors of the honey bee—structural features and phylogenetic analysis

The gene predicted as GB16571-PA in the A. mellifera genome showed a high degree of sequence similarity at the amino acid sequence level with vitellogenin receptors of other insects, such as the wasp N. vitripennis (64%), the ant S. invicta (63%), the cockroaches P. americana (57%), B. germanica (55%), L. maderae (55%) and the mosquito A. aegypti (55%). Since in silico analysis of the GB16571-PA amino acid sequence suggested that its 5’-end may have been predicted incorrectly we designed specific primers to determine the correct start codon by 5’-end sequencing. As a result, the predicted sequence (4860 bp) was extended by 312 bp.

Analysis of the deduced amino acid sequence defined this putative VgR of A. mellifera as a member of the LDL receptor family, which is characterized by a highly conserved arrangement of modular elements (Fig. 1A). For AmVgR protein two ligand-binding domains (LBD1 and LBD2) were predicted, with four Class A cysteine-rich repeats in the first domain and eight repeats in the second domain. The presence of only four Class A cysteine-rich repeats resembles most closely the VgR architecture of the ant S. invicta and of the wasp N. vitripennis. These hemonopeteran VgRs differ from those of other insects, like A. aegypti, P. americana, B. germanica and L. maderae, which possess five such repeats
(Sappington et al., 1996; Tufail and Takeda, 2005, 2007; Ciudad et al., 2006). Interestingly, hymenopterans share this character of four Class A repeats with the tick D. variabilis (Mitchell et al., 2007). Each ligand-binding domain is followed by an EGF precursor homology domain that contains two types of motifs, Class B repeats and YWXD repeats (Fig. 1A). After the second EGF precursor homology domain the putative AmVgR has an O-linked sugar region (LENMNTKLIFNSSLVIYKNESIRHQNGTLI) that is also present in N. vitripennis but is not found in S. invicta. The transmembrane domain (GIIITVLACIIIGSAYF) predicted by the PHDhtm Transmembrane Helices Prediction program (Combet et al., 2000) is followed by a cytoplasmic domain which contains a canonical clathrin-coated pit internalization motif (FXNPXY) (Rodenburg et al., 2006), that is also present in the cytoplasmic tail of the VgRs of S. invicta (Chen et al., 2004) and N. vitripennis.

The analysis of canonical motifs and domains in the putative lipophorin receptor predicted in the honey bee genome (GenBank accession no. XP_395858.3) also revealed the typical architecture of an LDLR family member, such as: (1) Class A cysteine-rich repeats, (2) EGF precursor homology domain with Y/FWT/VD repeats, (3) a serine and threonine-rich O-linked sugar region, (4) a hydrophobic transmembrane domain, and (5) a cytoplasmic
domain. Furthermore, the predicted LpR of *A. mellifera* has 76% and 74% similarity at the amino acid sequence level with the LpRs of *B. germanica* and *L. maderae*, 78% with *L. migratoria* and 75% with *G. mellonella*, revealing that these receptors are conserved.

This high degree of conservation of both honey bee receptors and their respective orthologs was reflected in the molecular phylogeny of selected LDLR family members. The insect VgRs form a distinct basal clade within the LDLRs, and within the insect VgRs the putative AmVgR groups together with the VgRs of the other hymenopterans (Fig. 1B). The predicted honey bee AmLpR forms a basal branch in the cluster containing the insect LpRs, and this cluster constitutes the sister group to the major branch of vertebrate LDLRs. This coherent separation of insect and vertebrate LDLRs is only interrupted by the branch composed of megalins which contains members of both clades.

### 3.2. Expression analysis of the putative honey bee gene *Amvgr* using semi-quantitative and quantitative PCR and in situ hybridization

The accumulating evidence for pleiotropic functions of vitello-genin in the context of honey bee sociobiology prompted us to investigate tissue-specific expression patterns of its putative receptor by semi-quantitative RT-PCR. Using specific primers we could detect *Amvgr* transcripts not only in eggs and in the female ovaries, but also in fat body, in the head and hypopharyngeal glands. A very low level of *Amvgr* transcripts was detected in midgut samples (Fig. 2A). Even though the strongest expression was observed in worker ovaries and in early embryos sampled 0–6 h after oviposition, the relatively widespread expression of the putative honey bee *Amvgr* is in accordance with the suggested pleiotropic functions of this protein.

*Amvgr* transcript levels were quantified in ovaries of workers kept in the presence or absence of a queen, and in ovaries of virgin and egg-laying queens. A drastic upregulation in *Amvgr* expression was seen in the ovaries of queens that had just started to lay eggs. Fig. 2B shows that levels of *Amvgr* transcripts increased in the ovaries of egg-laying queens in comparison to virgin queens. A similar result was obtained for worker ovaries. In the presence of the queen, workers showed very low levels of *Amvgr* expression in their ovaries (Fig. 2C). However, expression levels were clearly upregulated as soon as the ovaries showed signs of activation in queenless workers, particularly at the transition from stage I to stage II type ovaries when the amount of the mRNA increased. As outlined above, stage II ovaries contain previtellogenic to early vitellogenic follicles. There was no apparent difference in *Amvgr* expression between stage II and the more advanced stage III ovaries (Fig. 2D). These qPCR results confirmed the expected association between *Amvgr* expression and the reproductive status of a female honey bee, independent of whether it is a queen or a worker.

![Expression analysis of the putative honey bee vitellogenin receptor *Amvgr*.](image)

**Fig. 2.** Expression analysis of the putative honey bee vitellogenin receptor *Amvgr*. (A) Tissue-specific expression analyzed by RT-PCR from RNA extracts of newly laid eggs (E; embryos 0–6 h), and from head (H), fat body (FB), hypopharyngeal gland (HG), midgut (MG) and ovary (OV) of adult workers. Amplification of a cytoplasmic actin gene (*act*) served as loading control. C (−) is the negative control (no template), and M is the molecular weight marker (100 bp ladder). (B) *Amvgr* transcript quantification in ovaries of virgin and egg-laying queens; (C) Relative quantification of *Amvgr* mRNA in inactive ovaries of workers kept in the presence of a queen (queenright) and in inactive and active ovaries of workers kept for 18 days under queenless conditions; (D) *Amvgr* expression in queenless worker ovaries classified according to the degree of activation: (I) inactive ovaries, (II) ovaries exhibiting initial stages of follicle growth, and (III) ovaries with large vitellogenic follicles. Relative expression values were calculated according to the ΔΔCt method using a cytoplasmic actin gene as control gene. Each bar corresponds to a single biological sample represented as the mean ± S.D. of its technical replicates.
For a global comparison of reproductive versus non-reproductive females we grouped the quantitative PCR results for all females with active ovaries and compared them with the results for females with inactive ovaries. This analysis showed a clear statistical difference between the two groups (Mann–Whitney rank sum test, \( n = 16, P < 0.001 \)) indicating upregulation of Amvgr expression as females become reproductive.

This relationship is further supported by the results of in situ hybridization experiments (Fig. 3). In the honey bee ovary, the ovarioles are of the polytrophic meroistic type, but, in comparison to Drosophila, their terminal filament and germarium is much elongated (see Fig. 3A for a schematic representation). In these ovarioles, putative AmVgR encoding mRNA was exclusively detected in germ line cells (oocytes and trophocytes) with expression levels accompanying increasing follicle growth (Fig. 3B). As soon as the follicles separate from the gerarium and become divided into a trophic and an egg chamber, Amvgr expression appears to be strongly induced in the trophocytes, showing an apical to basal gradient in the trophic chamber (Fig. 3B and E). At this stage and also in the later vitellogenic stages, Amvgr transcripts are homogeneously distributed in the cytoplasm of the oocyte and are absent in the overlaying follicle epithelial cells.
In these vitellogenic follicles, the oocyte exhibits strong labeling, in accordance with the expectedly active receptor-mediated endocytosis of vitellogenin from the hemolymph (Fig. 3C). Surprisingly, we could detect Amvgr transcripts in oocytes already as soon as they become clearly distinct in the lower portion of the germarium (Fig. 3D). The specificity of the labeling was confirmed by hybridization with an Amvgr sense probe (Fig. 3F and G).

3.3. Expression analysis of the putative honey bee gene Amlpr using semi-quantitative and quantitative PCR

The transcriptional profile of the putative honey bee Amlpr gene was monitored by semi-quantitative RT-PCR. As shown in Fig. 4A, Amlpr transcripts were detected in various tissues of adult bees and also in embryos. In RNA samples obtained from heads of adult workers, but not in other tissues (analyzed in Fig. 4A), we detected two amplification products, one of 468 bp detected in all other samples, and a second smaller one of 381 bp (Fig. 4B). Sequence analysis of these PCR products revealed that the 381 bp fragment lacked a stretch of 87 bp that codifies for 29 amino acid residues in the O-linked sugar domain. Alignment of the deduced amino acid sequences of the Amlpr+ (variant form with a complete O-linked sugar domain; GenBank accession no. DQ091184) and Amlpr- (variant form lacking the 29 amino acid residues of the O-linked sugar domain; GenBank accession no. DQ091183) is shown in Fig. 4C.

When investigating the modulation of Amlpr transcription in the context of the reproductive cycle we observed that Amlpr expression is high in inactive ovaries of virgin queens and becomes reduced by nearly 75% in active ovaries of egg-laying queens (Fig. 4D). Similarly, in inactive ovaries of workers, Amlpr transcript...
levels were higher than in active ovaries (Fig. 4E). Therefore, for both females, higher levels of Amlpr transcripts were related to non-reproductive status. Interestingly, in the absence of the queen, the orphan workers fall into two very distinct groups in terms of Amlpr expression in their ovaries (Fig. 4E). Workers that did not activate their ovaries showed even higher levels, whereas in workers with activated ovaries Amlpr expression was reduced to very low levels. The analysis of Amlpr transcription in relation to the degree of ovarian activity in orphan workers (Fig. 4F) showed a clear negative relationship between Amlpr expression and reproductive state, exhibiting high levels in ovaries that contained only early follicles, followed by a gradual decline as follicles become previtellogenic and vitellogenic.

Again, for a global comparison of reproductive versus non-reproductive females we grouped the quantitative PCR results for all females with active ovaries and compared them with the results for females with inactive ovaries. This analysis showed a clear statistical difference between the two groups (t-test, t = 2.231, d.f. 14, P = 0.043) indicating downregulation of Amlpr expression as females become reproductive.

4. Discussion

In the present study we identified putative vitellogenin and lipophorin receptors in the honey bee, A. mellifera, and investigated their expression with respect to tissue specificity and in the context of female reproduction, which is at center stage of sociality in these hymenopterans. The deduced amino acid sequence of both predicted receptors shows the typical domains encountered in the LDLR superfamily. Multiple alignment of members of this family in insects, other arthropods, vertebrates and a nematode as an out group, revealed a series of interesting aspects in the molecular evolution of the LDLR superfamily. The putative A. mellifera VgR and LpR both fall into strongly supported clusters of their corresponding insect receptor families. The insect VgRs are widely separated from the vertebrate VgRs, the latter clustering within the remainder of the vertebrate lipoprotein receptors. Interestingly, the sister group to these vertebrate lipoprotein receptors is the insect lipoprotein receptor branch, and together they form a strongly supported cluster (100% bootstrap value).

4.1. Expression pattern of the putative vitellogenin receptor in female honey bees

In most insects, vgr expression is highly tissue-specific and vgr transcript have exclusively been detected in the ovaries (Sappington et al., 1996; Schonbaum et al., 1995; Chen et al., 2004; Tufail and Takeda, 2005, 2007; Ciudad et al., 2006). This stands in contrast with observations in vertebrates where vgr expression is cyclic and tightly regulated by ecdysteroids or juvenile hormone (for review see Raikhel et al., 2005, 2007; Ciudad et al., 2006). This stands in contrast with observations in vertebrates where vgr expression is cyclic and tightly regulated by ecdysteroids or juvenile hormone (for review see Raikhel et al., 2005, 2007; Ciudad et al., 2006). This stands in contrast with observations in vertebrates where vgr expression is cyclic and tightly regulated by ecdysteroids or juvenile hormone (for review see Raikhel et al., 2005, 2007; Ciudad et al., 2006).

Amvgr expression is strongly correlated with follicle development in both female castes, and in this respect it is similar to the mosquito A. aegypti. In the latter, vgr transcript levels in the ovaries rapidly increase after adult eclosion and continue to rise as the ovaries become vitellogenic, reaching peak levels 24 h after a blood meal (Cho and Raikhel, 2001). In cockroaches on the other hand, VgR orthologs are expressed in all stages of follicle development, with highest level observed in the immature ovaries of nymphs (Ciudad et al., 2006) and in ovaries containing early previtellogenic oocytes (Tufail and Takeda, 2005, 2007).

This apparent variability in the association of vgr expression with the female reproductive cycle is further illustrated by expression analysis in the ant S. invicta, where higher transcript levels were reported for winged virgin females than for egg-laying queens (Chen et al., 2004). In D. melanogaster, mRNA and protein of the corresponding yolk protein receptor yolkless were detected very early during the development of the oocyte, long before vitellogenesis begins (Schonbaum et al., 2000). For the honey bee, the current findings on the spatial and temporal dynamics of Amvgr expression can be interpreted as correlating both with the novel functions of vitellogenin in the life cycle of workers, as well as with its ancient function in sustaining vitellogenic follicle growth.

In dipteran ovaries, Vg/YPR gene transcripts were localized in nurse cells and in the oocyte of each follicle (Sappington et al., 1996; Schonbaum et al., 2000; Cho and Raikhel, 2001). The latter study also showed that a transgenic yl was transcribed exclusively in nurse cells and that the yl transcript subsequently accumulates in the developing oocytes (Schonbaum et al., 2000). The gradient in Amvgr transcripts that we detected in the honey bee ovarioles would be consistent with this observation. The situation is different in the panoistic ovaries of P. americana (Tufail and Takeda, 2005) and B. germanica (Ciudad et al., 2006) where vgr transcription starts during the early steps of oocyte differentiation, long before it would be functionally required. In P. americana, vgr gene expression is high in immature oocytes, and this is followed by a decline in the transcript signal in more developed oocytes. The decrease in vgr transcript levels in mature oocytes was interpreted as a recycling of the functional receptor that was synthesized during the immature stages.

The in situ hybridization analysis in honey bee queen ovarioles may bridge some of the above listed apparent contradictions reported for vgr expression in insect ovaries. In contrast to the dipterans, and also cockroaches, where oogenesis is cyclic and tightly regulated by ecdysteroids or juvenile hormone (for review see Raikhel et al., 2005), this is not the case in honey bees. In the polytrophic meristic ovaries of the honey bee queen, oogenesis is turned on shortly after the mating flight and then continues uninterruptedly allowing the queen to produce up to 2000 eggs/day in the approximately 400 ovarioles of the two ovaries (reviewed in Hartfelder and Engels, 1998). The onset of follicular Amvgr expression was first detected in the lower germarium, where the oocyte assumes a basal position and becomes surrounded by its nurse cells in a comet-like arrangement (Tanaka and Hartfelder, 2004). Next, Amvgr transcription becomes highly activated in the trophocytes of previtellogenic follicles in an apical to basal gradient within each trophic chamber. Amvgr encoding mRNA then accumulates in the growing oocytes, probably as a result of active transport from the trophocytes into vitellogenic oocytes. In all stages of follicle development, Amvgr expression appears to be clearly restricted to the germ line.

Unquestionably, the evidence coming from similarity and expression analysis holds the possibility that, because of its extravarian expression, the gene that we annotated as Amvgr may not be the endogenous honey bee vitellogenin receptor but a related protein. Yet, the temporal and spatial expression pattern that we observed in the ovaries is strongly supportive.
4.2. Expression pattern of a putative lipophorin receptor in female honey bees

Due to the wider functionality of lipophorins it was not surprising to find also in the honey bee that a gene encoding a putative lipophorin receptor showed little tissue restriction. An aspect of interest was the detection of two Amlpr mRNA isoforms in heads of honey bee workers. These variant forms of a putative honey bee AmlPr differ in the O-linked sugar domain. The existence of splice variant forms of LpR was described in some insect species and was always found related to tissue and stage-specific expression. In A. aegypti, the two LpR splice variants found in fat body and ovary differ in their amino termini, ligand-binding domains and in the O-linked sugar domains (Seo et al., 2003). The LpR of G. mellonella also has two splice variant forms, one being the full length LpR, while the other lacks an 84 bp segment in the O-linked sugar domain (Lee et al., 2003). In B. mori, four isoforms of the lipophorin receptor were found, with one of these, LpR4, being specifically expressed in the brain and central nervous system (Gopalapillai et al., 2006). Also in B. germanica, two LpR isoforms differing from each other by an insertion/deletion of 24 amino acids in the O-sugar linked domain were obtained from fat body and ovaries (Ciudad et al., 2007). The tissue or stage-specific expression of LpR isoforms is clearly of interest, but without knowledge of the precise role of the O-linked sugar domain, the exact function of these splice variants is unclear.

With respect to the dynamics of Amlpr expression in the honey bee ovary, the general pattern is similar to that observed in G. mellonella, where lpr transcription was detected in immature follicles only (Lee et al., 2003). In B. germanica, mRNA levels of both LpR isoforms were found to increase in the fat body during the first vitellogenic cycle, but in the ovary they decreased (Ciudad et al., 2007). This pattern resembled the expression dynamics of the vitellogenin receptor in this cockroach (Ciudad et al., 2006), suggesting that the two receptors may be coregulated. However, this does not seem to be the case in the honey bee ovary where the transcription levels for Amvgr and Amlpr exhibit exactly opposite directionality.

In conclusion, our results on expression patterns of the two honey bee LDLR members show that their regulation in the reproductive cycle appears to follow opposing trends, the putative lipophorin receptor being upregulated as the ovaries are activated, whereas the putative lipophorin receptor becomes downregulated. This is the case in both castes, which otherwise are very different in their physiologies, especially with respect to the circulating vitellogenin titer (Hartfelder and Engels, 1998). Different from the vitellogenin titer which evolved into a phenotype similar to that of the Drosophila yokless mutant. FEBS Journal 273, 325–335.

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Appendix A. Supplementary data


References


