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Systemic RNAi of the cockroach vitellogenin receptor results in a phenotype similar to that of the Drosophila yolkless mutant

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Blattella germanica; panoistic ovaries; vitellogenin receptor; yolkless

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During vitellogenesis, one of the most tightly regulated processes in oviparous reproduction, vitellogenins are incorporated into the oocyte through vitellogenin receptor (VgR)-mediated endocytosis. In this paper, we report the cloning of the VgR cDNA from *Blattella germanica*, as well as the first functional analysis of VgR following an RNA interference (RNAi) approach. We characterized the VgR, VgR mRNA and protein expression patterns in pre-adult and adult stages of this cockroach, as well as VgR immunolocalization in ovarioles, belonging to the panoistic type. We then specifically disrupted *VgR* gene function using RNAi techniques. Knockdown of *VgR* expression led to a phenotype characterized by low yolk content in the ovary and high vitellogenin concentration in the haemolymph. This phenotype is equivalent to that of the yolkless mutant of *Drosophila melanogaster*, which have the *yl* (*VgR*) gene disrupted. The results additionally open the perspective that development genes can be functionally analyzed via systemic RNAi in this basal species.

From worms to chickens, vitellogenesis is one of the most emblematic processes related to reproduction in oviparous animals. By this process, yolk proteins produced by vitellogenic tissues (usually the fat body in insects and the liver in vertebrates) are taken up by the growing oocyte. Detailed descriptions of vitellogenesis have been reported in invertebrates, especially insects [1], and in vertebrates, in particular birds, frogs and fishes [2].

During vitellogenesis, vitellogenins are incorporated into the oocyte through a receptor-mediated endocytic pathway [3], and a key element in the whole process is the vitellogenin receptor (VgR). Quite unexpectedly, reported VgRs from insects, fishes, frogs and birds are homologous and belong to the same low-density lipoprotein receptor (LDLR) superfamily [4]. In insects, the VgR has been characterized from gene or cDNA sequencing in the fruit fly *Drosophila melanogaster* [5], the mosquito *Aedes aegypti* [6], the ant *Solenopsis invicta* [7] and the cockroach *Periplaneta americana* [8]. The structural conservation of the VgR in insects is even more surprising, as the ligand may vary depending on the group. Although the great majority of insects use vitellogenins as yolk precursors, exceptionally, *D. melanogaster* uses structurally unrelated yolk polypeptides (YP) for the same purpose [9]. Nonetheless, the YP receptor of *D. melanogaster* is a VgR homologous to the VgRs of other insects [10].

The conservation of VgR in insects is also surprising given the diversity of insect ovariole structure. Insects show two basic types of ovarioles: The most primitive, called panoistic, in which all oogonia develop into

Abbreviations
BgVgR, *Blattella germanica* vitellogenin receptor; dsRNA, double-stranded RNA; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; JH, juvenile hormone; LDLR, low density lipoprotein receptor; RNAi, RNA interference; VgR, vitellogenin receptor; YP, yolk polypeptides.

oocytes, is typical of primitive groups, occurring, for example, in most Polyneoptera. The other type, called meroistic, occurs in more modified insects, as in most Paraneoptera and Holometabola, and identifies ovarioles in which oogonia give rise to both oocytes and nurse cells [11]. Despite such ovariole diversity, which suggests a potentially parallel diversity in vitellogenic mechanisms, the VgR reported in insects with panoistic (*P. americana*) and meroistic (*S. invicta*, *D. melanogaster* and *A. aegypti*) ovarioles are homologous.

The question that arises is whether this degree of structural conservation of VgR across such diverse groups is paralleled by an equivalent degree of conservation of functional and regulatory mechanisms for the receptor. Developmental and regulatory studies of insect VgRs have been reported in *D. melanogaster* [5,12], *A. aegypti* [6,13] and *P. americana* [8]. However, functional studies involving loss-of-function approaches have only been carried out in *D. melanogaster*, due to the inherent advantages offered by this species for genetic analysis. In this context, the female sterile mutation *yolkless*, which is characterized by containing very little yolk in the oocytes and by producing defective chorion layers, served not only to unravel key steps in the process of receptor-mediated endocytosis [14], but also to characterize the *D. melanogaster* VgR encoded by the *yolkless* gene [5], and it is still a useful tool to study the regulation of the *VgR* in this species [12].

Functional studies involving loss-of-function approaches in nondrosophilid insects having primitive panoistic ovarioles, such as cockroaches, have traditionally been hampered because they are not easily amenable to genetic transformation. However, the RNA interference (RNAi) techniques, by which a target mRNA is eliminated after treatment with a double-stranded RNA (dsRNA) homologous to it [15], has opened a new avenue to perform gene function analyses in nondrosophilid species. For example, RNAi has been used in the cockroach *P. americana* to analyze the function of the homeotic gene *engrailed* in relation to the control of axon pathfinding and synaptic target choice in neurons of the cercal sensory system [16]. In this paper, we report the first functional analysis of *VgR* using RNAi. As an experimental subject, we used *Blattella germanica*, a cockroach with panoistic ovarioles that oviposits in an ootheca which is transported by the female until egg hatching, and whose vitellogenesis has been thoroughly studied [17–19]. We first cloned and characterized the *VgR* cDNA of this species, then we determined the developmental expression pattern in the last instar nymph pre-adult and in the adult, and we immunolocalized the VgR in the ovariole in different physiological situations. Finally, we developed the RNAi experiments *in vivo*, which allowed us to efficiently and specifically disrupt *VgR* gene function.

Results

Cloning and sequencing of *B. germanica* VgR

Following a degenerate RT-PCR approach, a 980 basepair fragment of *B. germanica* vitellogenin receptor (*BgVgR*) was cloned. The sequence was then completed using a λZapII cDNA library from *B. germanica* adult ovaries as a template, and following a seminested PCR approach, using primers drawn from the 980 basepair fragment of the *BgVgR*, and λZap-specific primers. This procedure led to obtain a 5768 basepair sequence (GenBank accession number AM050637) with an open reading frame of 5457 basepairs encoding a protein of 1819 amino acids with a predicted molecular mass of 202.3 kDa and an isoelectric point of 4.94. The 3′-UTR region had 205 basepairs, and a polyadenylation degenerated signal was located 38 basepairs downstream from the stop codon. After the first methionine, which is preceded by a series of stop codons, a putative peptide signal is located between positions 1–25, with a probable cleavage site within residues 25 and 26 (predicted with the SIGNAL IP 3.0 program [20]).

The organization of the BgVgR amino acid sequence indicates that it is a member of the LDLR superfamily receptors, characterized by the conservation of modular elements (Fig. 1A). BgVgR has two ligand-binding domains with five and eight class A cysteine-rich repeats, respectively (Fig. 1A). Each ligand binding domain is followed by an epidermal growth factor (EGF) precursor homology domain that contains two types of motifs, the class B repeats, with six cysteine each, and the YWXD repeats, also in a number of six (Fig. 1A). Following the second EGF precursor homology domain, there is an *O*-linked sugar region, very rich in serine, a transmembrane region between amino acids 1690–1705 and, finally, a cytoplasmic domain. This cytoplasmic domain includes a region homologous to the internalization consensus sequence FXNPXF in position 1722, and a motif containing LI five positions after an acidic residue (DGKVLI, residues 1762–1768) that can serve as alternative internalization signal. Possible sites for co- and post-translational modification other than the *O*-linked sugar region, include 13 N-linked glycosylation sites (having the consensus motif NXS/T) and 92 putative phosphorylation sites (predicted with

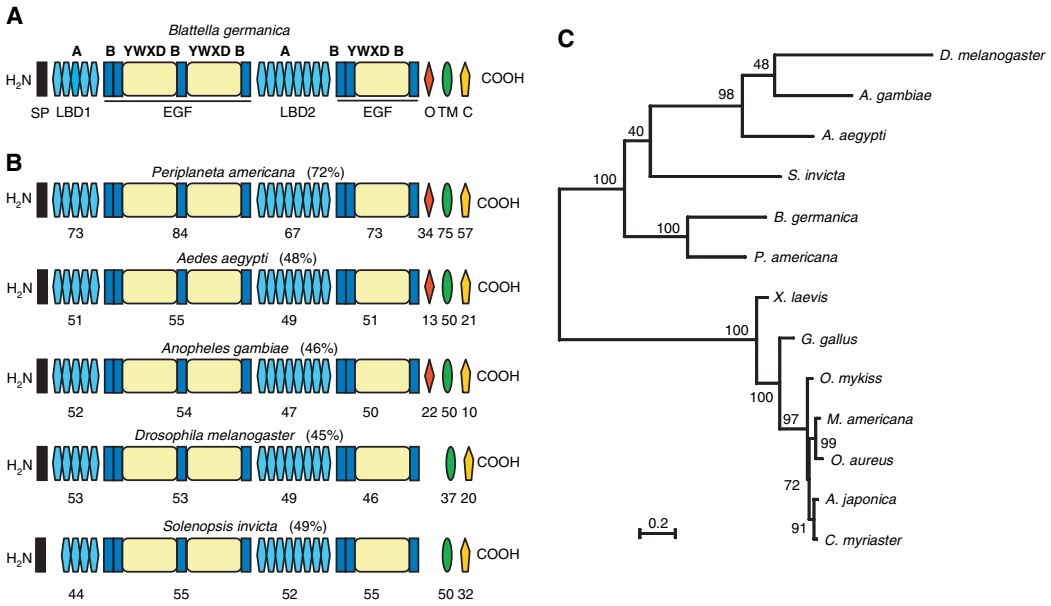

Fig. 1. Vitellogenin receptor of *B. germanica*. (A) Organization of *B. germanica* VgR showing the characteristic domains of an LDL receptor. (B) Comparison of modular domains between different insect VgRs. Percentage of similarity with respect to *B. germanica* sequence is indicated (overall similarity indicated beside the species name, with domain similarity below the corresponding domain). A, Class A cysteine-rich repeats; B, class B cysteine-rich repeats; C, cytoplasmatic domain; EGF, epidermal growth factor precursor homology domain; LBD, ligand binding domain; O, *O*-linked sugar domain; SP, signal peptide; TM, transmembrane domain. (C) Phylogenetic tree showing the position of *B. germanica* VgR with respect to other insect and vertebrate VgRs. The tree was constructed based on the maximum-likelihood method. Branch lengths are proportional to sequence divergence. The bar represents 0.2 differences per site. Bootstrap values are shown in the node of clusters. The vertebrate cluster was used as out-group. See generic names in the text.

the NETPHOS program [21]) on serine and threonine residues.

Sequence comparisons and phylogenetic analysis

The deduced primary structure of BgVgR was compared with VgRs of the cockroach *P. americana*, the mosquitoes *A. aegypti*, *Anopheles gambiae*, the fruit fly *D. melanogaster*, and the ant *S. invicta* (Fig. 1B). As expected, BgVgR was most similar to *P. americana* VgR (72% overall similarity, with 84% similarity when comparing the first EGF precursor homology domain). Similarity among VgRs of the other insects (Diptera and Hymenoptera) was rather low (between 46 and 49% overall similarity, and from 53 to 55% when comparing the first EGF precursor homology domain) (Fig. 1B). Maximum-likelihood analysis of these insect VgR sequences, using the VgR of vertebrates as an out-group, generated the tree shown in Fig. 1(C), whose topology approximately follows the current phylogeny of these species. *D. melanogaster* has the longest branch length, suggesting a faster rate of divergence with respect to other sequences. Vertebrate branches are much shorter, indicating the great conservation of these sequences.

BgVgR developmental patterns

RT-PCR studies in different adult female tissues and in RNA from whole male extracts, showed that *BgVgR* expression is restricted to ovarian tissues (Fig. 2).

The developmental expression pattern of *BgVgR* mRNA was studied in ovaries of last instar nymphs

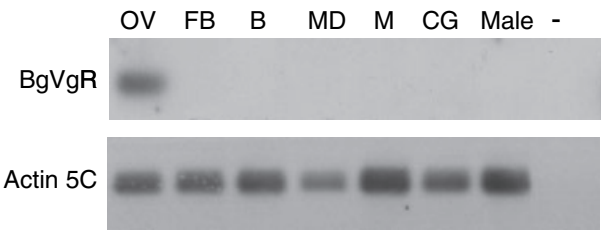

Fig. 2. Expression of *B. germanica* VgR (BgVgR) in different adult tissues. RT-PCR was carried out with total RNA isolated from 3-day-old female ovary (OV), fat body (FB), brain (B), midgut (MD), extensor muscle (M), colleterial gland (CG) and from whole male extracts (Male). The last lane (–) represents total RNA without reverse transcription, indicating that there was no genomic contamination. *B. germanica* actin5C levels were used as a reference.

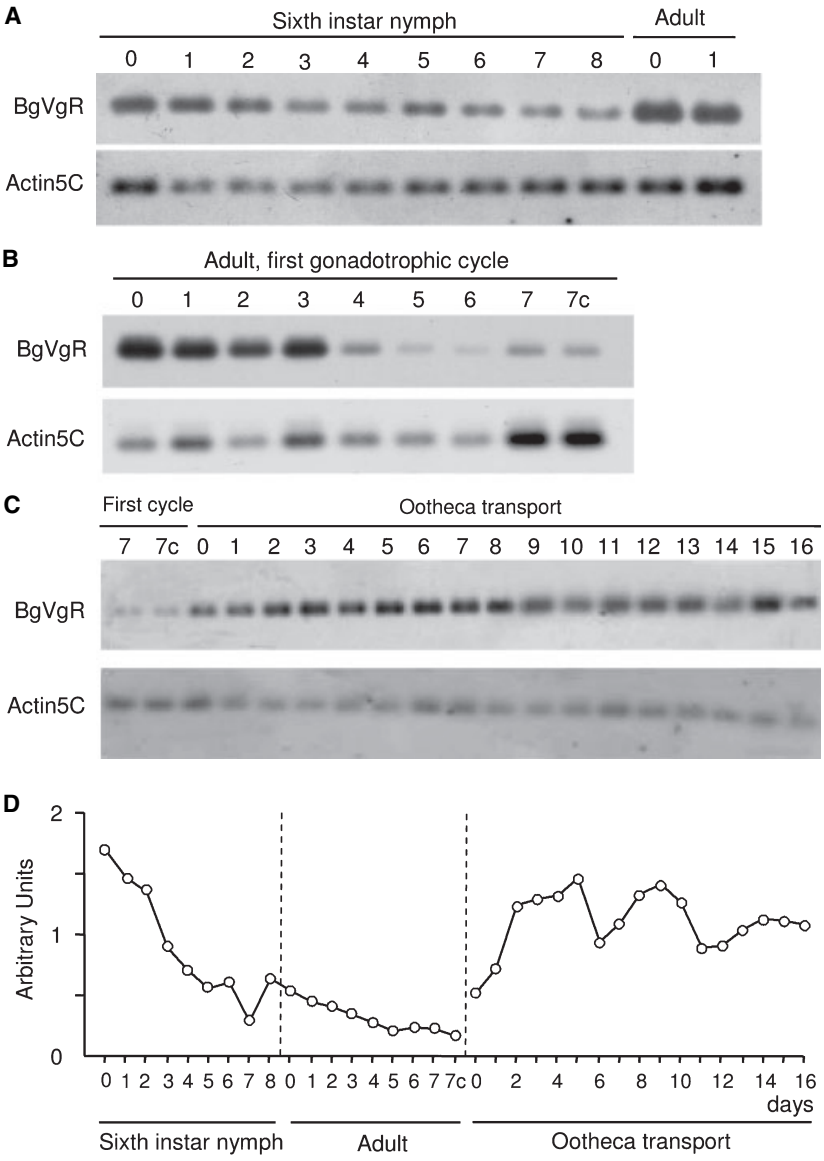

Fig. 3. *BgVgR* mRNA expression in ovaries of *B. germanica*. (A) Sixth instar nymphs. (B) Adults in the first gonadotrophic cycle. (C) Adults during the period of ootheca transport. 7c, 7-day-old adult with the basal oocyte with chorion layers present. (D) Densitometry values of BgVgR RT-PCR bands corrected with respect to actin5C bands ($n = 3$).

and adult females during both the first gonadotrophic cycle and the period of ootheca transport. Ovarian *BgVgR* mRNA levels appeared high at the beginning of the last nymphal instar, steadily declining along the instar, and reaching the lowest values of the instar before the imaginal moult (Fig. 3A). In the adult, mRNA levels remained low or even still decreased until oocyte chorionation and oviposition (Fig. 3B). After oviposition, mRNA levels rapidly increased, remaining high, with some fluctuations, during the entire period of ootheca transport (Fig. 3C).

Developmental expression patterns of *BgVgR* in terms of protein were also studied. BgVgR levels were very low at the beginning of the last nymphal instar, but increased steadily until the imaginal moult (Fig. 4A). In the adult, BgVgR levels remained high, while exhibiting some fluctuations, and reached their highest values just before oviposition (Fig. 4B). After oviposition, BgVgR levels suddenly dropped, remaining very low during the period of ootheca transport (Fig. 4C).

BgVgR immunolocalization

BgVgR localization in ovaries was examined by immunofluorescence in last instar nymphs, as well as in the adults. In the first days of last instar nymphs, BgVgR protein was detected as a very faint fluorescence, close to background level, evenly distributed in the oocyte cytoplasm (Fig. 5A,B). On days 4–5, however, BgVgR

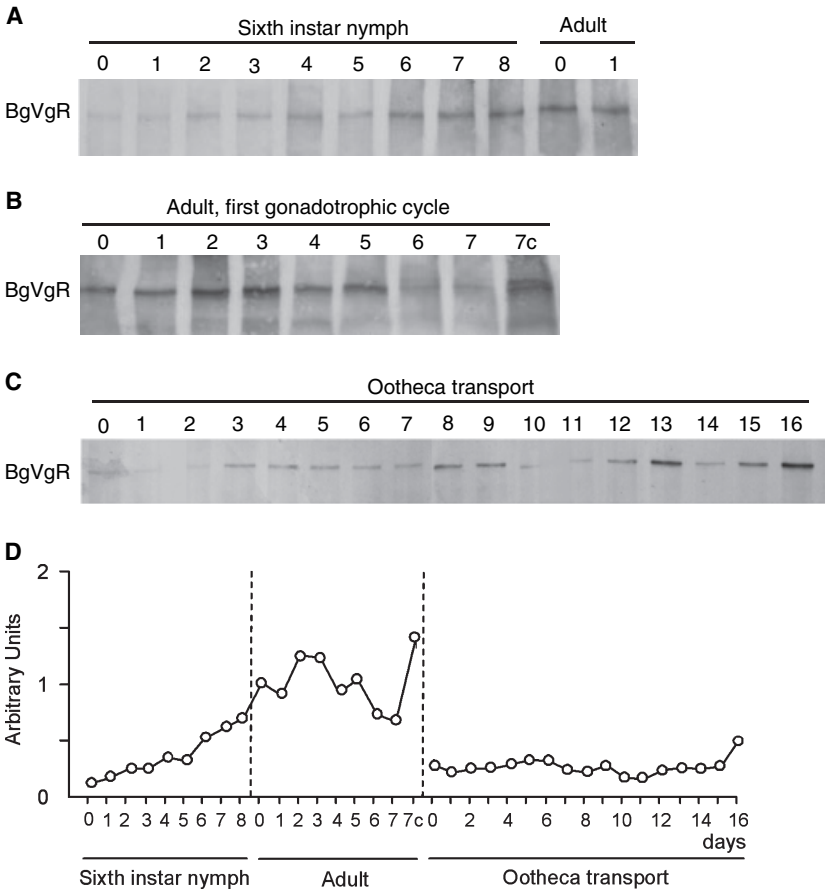

Fig. 4. BgVgR protein expression in ovaries of *B. germanica*. (A) Sixth instar nymphs. (B) Adults in the first gonadotrophic cycle. (C) Adults during the period of ootheca transport. 7c, 7-day-old adult with the basal oocyte with chorion layers present. (D) Densitometry values of BgVgR western blot bands (n = 3). 0.1 ovary equivalents were loaded in each lane.

became clearly visible accumulating in the cortex of the basal oocyte, whereas in secondary oocytes it remained evenly distributed in the cytoplasm (Fig. 5C,D). This localization pattern was maintained in the adult (Fig. 5E,F) until days 6–7, that is, 1–2 days before oviposition, when BgVgR also accumulates in the cortex of the subbasal oocyte. When this subbasal oocyte occupied the basal position just after oviposition, the BgVgR had clearly accumulated in the cortex (Fig. 5G,H).

Silencing *BgVgR* expression by RNAi

Silencing the *BgVgR* gene would presumably lead to a phenotype characterized by low ovary vitellin content, and high haemolymph vitellogenin content. In an initial series of RNAi experiments, 5 μg of dsBgVgR were injected in freshly emerged *B. germanica* adult females, and ovaries were dissected 4 and 6 days later. In comparison with specimens treated with dsControl, silencing effects were detectable (especially on day 6), but proved weak, in terms of BgVgR reduction in the ovary, vitellin depletion in the basal oocyte, and vitellogenin accumulation in the haemolymph (results not shown). Although weak, the effects were clearer on day 6 than on day 4, which led us to carry out the RNAi treatment earlier. Thus, newly emerged last instar nymphs were treated with 5 μg of dsBgVgR or with the same amount of dsControl. In general, treated and control nymphs molted to adults 8 days later, and were dissected on days 0, 4 or 6 after adult emergence. Western blot analysis indicated that BgVgR levels were dramatically reduced in ovaries of dsBgVgR-treated females (Fig. 6A). Moreover, these specimens had smaller basal oocytes (Fig. 6B), with lower vitellin contents (Fig. 6C) than controls. This was concomitant with clear protein accumulation in the haemolymph (Fig. 6D), principally vitellogenin, as shown by western blot (Fig. 6E). All these effects were clearer on day 6 than on day 4 of adult life. On day 6, the vitellogenin accumulated in the haemolymph of dsBgVgR-treated was processed in a similar manner to that of vitellin in the ovary of control specimens (Fig. 6E). Immunolocalization studies in dsBgVgR-treated females revealed that in 6-day-old adult females, BgVgR does not accumulate in the cortex at all (Fig. 6F,G). Conversely, a

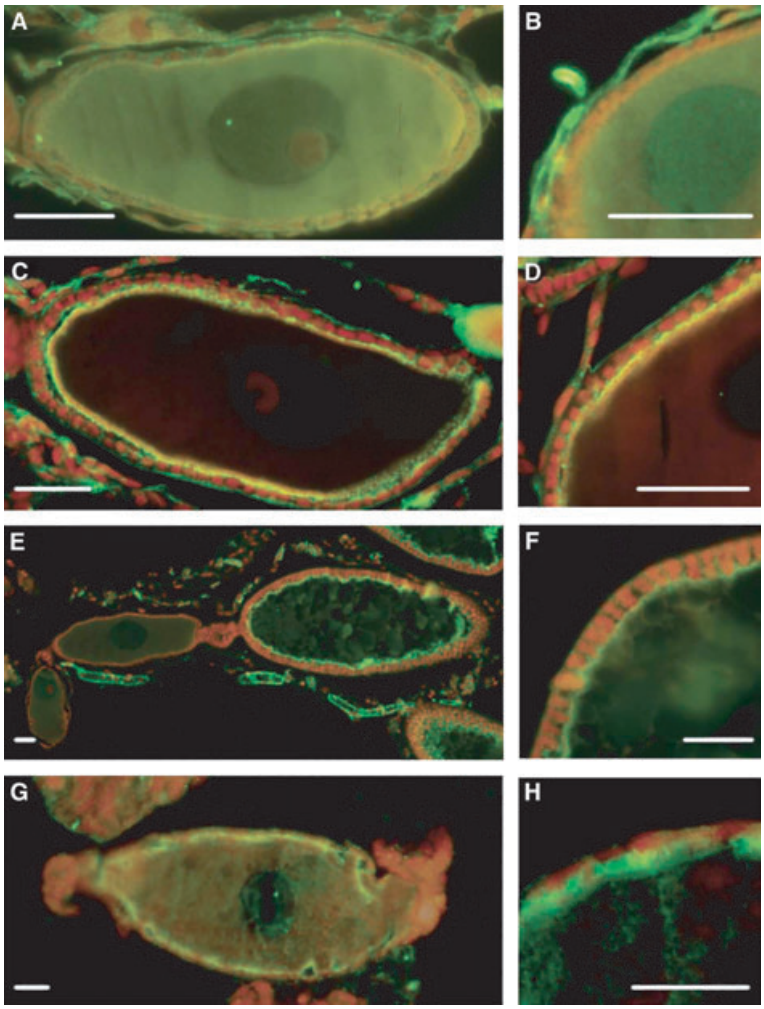

Fig. 5. Immunolocalization of BgVgR in ovaries of *B. germanica*. (A,B) Oocytes from 2-day-old, sixth instar nymphs; BgVgR does not accumulate in the cortex of basal oocytes. (C,D) Oocytes from 5-day-old, sixth instar nymphs; BgVgR accumulates in the cortex of basal oocytes. E-F. Oocytes from 3-day-old adult females showing BgVgR accumulated in the cortex of basal oocytes. (G,H) Basal oocytes of an adult female on the first day of the period of ootheca transport, showing BgVgR in the cortex. Scale bars: 50 μm.

clear cortical accumulation of BgVgR is observed in dsControl specimens (Fig. 6H,I). All dsBgVgR-treated females ($n = 12$) mated and had spermatozoids in the spermathecae. However, they resulted sterile, either not producing ootheca (17%), or producing a small ootheca (83%) containing between 6 and 18 nonviable eggs.

Discussion

We have characterized the cDNA of *BgVgR*, and the deduced amino acid sequence. As expected, the BgVgR is organized according to the modular elements of VgRs (Fig. 1A) and, in general, to receptors belonging to the LDLR superfamily [10]. Sequence comparisons with other VgRs revealed a high similarity (72%) with the VgR of the cockroach *P. americana*, and a moderate similarity (around 45%) with VgRs of holometabolous insects (Fig. 1B). Phylogenetic analysis showed that the species cluster approximately as in current phylogenies, and that *D. melanogaster* VgR seems to have a faster rate of divergence with respect to other insect VgRs (Fig. 1C), which could be related to the different ligand (YP) used by this species.

Expression studies in different tissues and in both sexes, indicates that *BgVgR* is specifically expressed in ovaries (Fig. 2). Developmental patterns show that *BgVgR* mRNA levels are high at the beginning of sixth instar nymph, decline thereafter, remaining low during the first reproductive cycle in the adult stage, and recovering high relative values during the period of ootheca transport (Fig. 3). This pattern is only slightly different to that of *P. americana*, in which *VgR* mRNA levels are relatively high at the beginning of the adult stage, at previtellogenic period, declining on day 3 after the adult emergence, and remaining low during the vitellogenic phase [8]. In the ant *S. invicta*, *VgR* mRNA levels are higher in virgin alate females than in fully vitellogenic queens [7]. Similar patterns of high *VgR* mRNA levels in

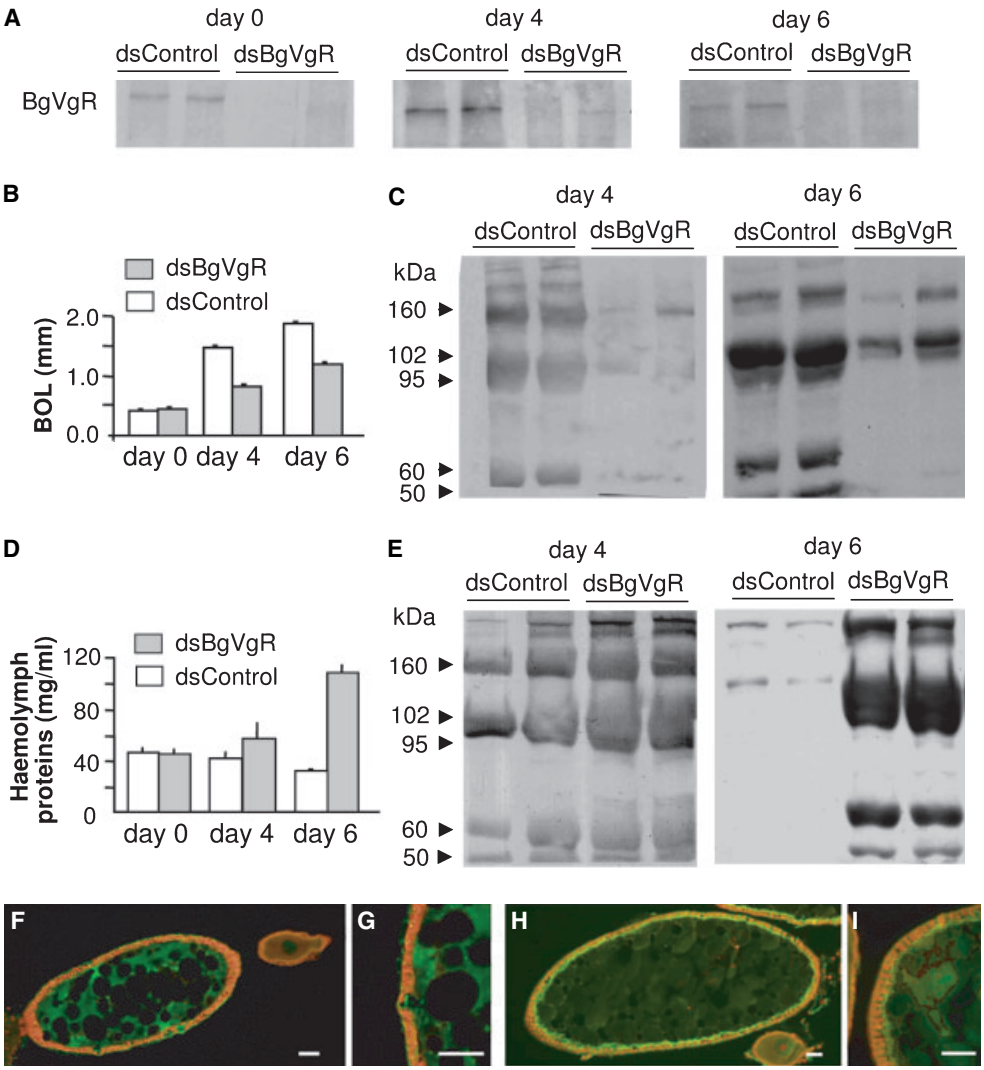

Fig. 6. Silencing BgVgR expression in *B. germanica*. dsBgVgR or dsControl was injected in newly emerged sixth instar nymphs and dissections were made just after adult emergence (day 0) and 4 and 6 days later. (A) Western blot showing the expression of *BgVgR* in the ovary. (B) Basal oocyte length (BOL). (C) Vitellin in ovaries from 4- and 6-day-old females. The indicated bands correspond to vitellogenin-vitellin subunits. (D) Haemolymph protein content. (E) Haemolymph vitellogenin. The indicated bands correspond to vitellogenin–vitellin subunits. The right gel (day 6) was subexposed to show a clearer pattern of dsBgVgR-treated specimens. (F,G) Immunodetection of BgVgR in ovaries of 4-day-old females that had been treated with dsBgVgR. (H,I) Immunodetection of BgVgR in ovaries of 4-day-old females that had been treated with dsControl. Scale bars: 100 μm. In A, C and E, 0.1 ovary equivalents were loaded in each lane.

non reproductive stages, and low or very low mRNA levels during full vitellogenesis is found not only in insects, but also in oviparous vertebrates, like chickens [22] and rainbow trout [23]. The most divergent pattern is exhibited by the mosquito *A. aegypti*, in which *VgR* mRNA starts to rise one day after the adult moult, continues to increase dramatically during the vitellogenic period, and then peaks one day after the blood meal [24]. This particular pattern is surely related to the haematophagous regime and anautogenic features of this species.

The BgVgR protein pattern (Fig. 4D) is almost complementary to that of *BgVgR* mRNA (Fig. 3D). Increases in protein levels and decreases in mRNA occur towards the last third of the last instar nymph, and this is concomitant with the imaginal moult peak of ecdysteroids, which is produced in the absence of JH [25]. This coincidence suggests that the translation of *BgVgR* may be directly or indirectly determined by this endocrine context as a part of the functional metamorphosis occurring at the last molt. The low BgVgR proteins and high mRNA levels occurring during the

period of ootheca transport, characterized by low levels of both, JH and ecdysteroids ([26], and unpublished results of K. Treiblmayr, N. Pascual, X. Bellés and M.D. Piulachs), must be determined by a different regulatory mechanism. Expression of the BgVgR protein in the last instar nymph, when vitellogenesis has not yet begun [25], may represent an opportunistic strategy to proceed with vitellogenesis effectively from the very beginning. In *D. melanogaster*, VgR mRNA and protein are both expressed long before vitellogenesis begins [12].

Immunolocalization studies showed that the very few BgVgR present in the first days of the last instar nymph spreads into the oocyte cytoplasm (Fig. 5), albeit towards the mid-instar, concomitantly with a steady increase in BgVgR protein levels (Fig. 4), begins to accumulate in the cortex of the basal oocyte (Fig. 5). The adult shows a similar pattern, although towards the end of the first gonadotrophic cycle, the BgVgR begins to accumulate also in the cortex of the subbasal oocyte, which will become the basal oocyte after oviposition and during the period of ootheca transport. Although the levels are low during this period (Fig. 4), BgVgR accumulates in the cortex (Fig. 5). In previtellogenic *A. aegypti*, *D. melanogaster* and *P. americana*, the VgR spreads over the oocyte cytoplasm, but when vitellogenesis starts it accumulates in the cortex [8,12,13]. This suggests that VgR re-localizes to the cortex before the onset of vitellogenesis through a regulated mechanism. This phenomenon has also been reported in chickens [27]. In *B. germanica*, BgVgR localizes in the cortex as soon as it is synthesized in mid- last instar nymph, which suggests that protein sorting in the cortex is spontaneous and follows the universal pathway involving the exocyst complex [28].

Finally, we developed a reliable RNAi protocol to disrupt *BgVgR* gene function in *B. germanica*. RNAi treatment with dsBgVgR impaired vitellogenin uptake into basal oocytes, whereas this protein accumulated in the haemolymph (Fig. 6). In the haemolymph of dsBgVgR-treated, the vitellogenin accumulated so dramatically that on day 6 it was processed as it is in the ovary, a syndrome that had been previously observed in ovariectomized females [18]. This phenotype supports the notion that the isolated cDNA does indeed correspond to a functional *VgR* of *B. germanica*, and is equivalent to that of the *yolkless* mutant of *D. melanogaster* [14], which have the *VgR* (or *yl*) gene, mutated [5]. Yolkless mutants of *D. melanogaster* have much less yolk in their oocytes and do not present the VgR localized in the cortex [12]. The use of *D. melanogaster* yolkless mutants facilitated the unraveling of key steps involved in receptor-mediated endocytosis in meroistic oocytes [12,14]. Moreover, RNAi technique could extend this type of gene function analysis not only to the study of VgR in panoistic oocytes, but also to other genes in insect species not easily amenable to genetic transformation. With some 30 million non-drosophilid insect species on Earth [29], exploring gene function with RNAi appears a very worthwhile pursuit.

Experimental procedures

Insects

Freshly ecdysed sixth (last) instar nymphs or adult females of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% relative humidity. Dissections and treatments were carried out on carbon dioxide-anaesthetized specimens.

Cloning and sequencing

Degenerate primers based on conserved sequences of the VgR ligand binding domain of *A. aegypti* and *D. melanogaster* were used to obtain a *B. germanica* homologue cDNA fragment by PCR amplification, using cDNA template generated by reverse transcription from polyA$^+$ RNA from 3-day-old adult ovaries. The primers were as follows: forward 5′-GAYGGNDTNGAYGAYTGYGG-3′; and reverse 5′-ARYTTRGCATCBACCCARTA-3′. The amplified fragment (980 basepair) was subcloned into the pST-Blue^{TM}-1 vector (Novagen, Madison, WI, USA) and sequenced. To complete the sequence, a λZapII Express library generated from *B. germanica* ovaries was used as a template for seminested PCR, using specific primers based on the 980 basepair cloned fragment, and λZap-specific primers, as previously described [30]. The PCR products were analyzed by agarose gel electrophoresis, cloned into the pSTBlue^{TM}-1 vector, and then sequenced.

Sequence comparisons and phylogenetic analyses

Sequences of VgRs were obtained from GenBank. These included the insects *D. melanogaster* (AAB60217), *A. gambiae* (EAA06264), *A. aegypti* (AAK15810), *S. invicta* (AAP92450) and *P. americana* (BAC02725), and the vertebrates *Anguila japonica* (BAB64337), *Conger myriaster* (BAB64338), *Oncorhynchus mykiss* (CAD10640), *Oreochromis aureus* (AA027569), *Morone americana* (AA092396), *Xenopus laevis* (AAH70552), and *Gallus gallus* (NP_990560). Protein sequences were aligned with that obtained for *B. germanica* (BgVgR) using CLUSTALX (ftp.ebi.ac.uk). Poorly aligned positions and divergent regions were

eliminated by using GBLOCKS 0.91b (http://molevol.ibmb.csic.es/Gblocks_server/) [31]. The resulting alignment was analyzed by the PHYML program [32] based on the maximum-likelihood principle with the amino acid substitution model. Four substitution rate categories with a gamma shape parameter of 1.444 were used. The data was bootstrapped for 100 replicates using PHYML.

RT-PCR/Southern blot analyses

Profiles of BgVgR mRNA were obtained using RT-PCR followed by Southern blotting with a specific probe. Total RNA was isolated from four to six ovary pair pools from different developmental stages using the General Elute Mammalian TotalRNA kit (Sigma, Madrid, Spain). A 300 ng portion of each RNA extraction was DNAse treated (Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad CA, USA) and random hexamers (Promega). To study *BgVgR* mRNA patterns, these cDNA samples were subjected to PCR with a number of cycles within the linear range of amplification, with 35 cycles at 94 °C (30 s), 62 °C (30 s) and 72 °C (1 min). The BgVgR primers were as follows: forward 5′-CCA AGT GTA CAT TAT ATC CCA CCT G-3′; and reverse 5′-GAA CTA CGT ACA ATT GCT TCT TCT CC-3′. As a control, the same cDNAs were subjected to RT-PCR/Southern blotting with a primer pair specific to *B. germanica* actin5C [33]. cDNA probes for Southern blot analyses were generated by PCR with the same primer pairs, using plasmid DNA containing the corresponding cDNA clones as a template. The probes were labeled with fluorescein by the Gene Images random prime-labeling module (Amersham Biosciences, Barcelona, Spain). RT-PCR followed by Southern blotting of total RNA without reverse transcription was carried out in parallel as control for genomic contamination.

BgVgR antibody

A 576 basepair DNA fragment (from amino acid 703 to 894) corresponding to the EGF-like domain of *BgVgR* (a domain which is exclusive of insect *VgR*s) was chosen to produce a BgVgR recombinant fragment and to generate the corresponding polyclonal antibody. The PCR amplified fragment was cloned into the pSTBlue^{TM}-1 vector and sequenced. The insert was directionally subcloned into pET28a(+) (Novagen), using *EcoRI* and *HindIII* restriction sites. *E. coli* BL21 (DE3) plysS competent cells were used for plasmid transformation. The transformed bacteria were selected by screening the colonies on media containing 30 $\mu g \cdot mL^{-1}$ of kanamycin. Colonies were further analyzed by restriction enzyme digestion and PCR. Bacteria were grown until OD_{600nm} reached 0.8, and were then induced with 0.8 mM of IPTG for 3 h. The expressed protein was purified using a Ni-NTA (Qiagen, Hilden, Germany) column according to the manufacturer's instructions. The purified recombinant BgVgR fragment was quantified [34], and quality tested by SDS/PAGE 13% stained with $CuCl_2$. The 27.6 kDa band was excised and homogenized. Finally, it was resuspended in Ringer solution, emulsified with complete Freund's adjuvant, and used to boost New Zealand female rabbits. The resulting antibody recognized a band that fit with the predicted size of BgVgR (202 kDa). The antibody was further validated in the RNAi experiments.

Immunoblot analysis

Ovaries were dissected under Ringer solution, frozen with liquid N_2, and preserved at −70 °C until use. Haemolymph was collected with a calibrated micropipette applied to a cut femur. For each specimen, 1 μL haemolymph was dissolved in 50 μL sodium carbonate buffer 0.05 M (pH 9.6). For protein extraction, ovaries were homogenized in 100 μL of a buffer composed of 100 mM sucrose, 40 mM K_2HPO_4 pH 7.2, 30 mM EDTA, 50 mM KCl, 0.25% (v/v) Triton X-100, 10 mM DTT and 0.5 mM proteases inhibitor cocktail (Roche, Barcelona, Spain). After measuring the protein contents of homogenates [34], suramine (5 mM) was added to inhibit the binding of vitellogenin to its receptor. Homogenates were analyzed in 6.5% SDS/PAGE gels loading the same ovary equivalents per lane. To study haemolymph vitellogenin content, 0.25 μL haemolymph from individual females were analyzed in 6.5% SDS/PAGE. Gels were transferred to a nitrocellulose membrane (Protran, Schleicher and Schuell, Dassel, Germany) and incubated with BgVgR antibody (1 : 1000) or *B. germanica* vitellogenin antibody (1 : 20 000) [17] for 1 h, and were then processed for ECL western blotting (Amersham Biosciences), following the manufacturer's instructions.

Immunolocalization

Ovaries were fixed for 4 h in 4% paraformaldehyde in 0.2 M NaCl/P_i buffer (pH 6.8), serially dehydrated in ethanol, and embedded in paraffin. Sections of 8 μm were rehydrated, equilibrated in NaCl/P_i, saturated for 30 min at room temperature in 0.1% Triton X-100, 0.5% Bovine serum albumine (BSA) and 5% normal goat serum in NaCl/P_i, and incubated overnight at 4 °C with the primary anti-BgVgR antibody diluted 1 : 100 in a wet chamber. After three washes with NaCl/P_i, the sections were incubated with Alexa-Fluor 488 conjugated goat antirabbit IgG secondary antibody (Molecular Probes, Carlsbad, CA, USA; 1 : 400 in NaCl/P_i) for 2 h. Primary and secondary antibodies were suspended in the same buffer used for saturation. After three rinses (10 min each) in buffer, preparations were mounted in Mowiol medium (Calbiochem, Madison, WI, USA) and observed for immunofluorescence in an Axiophot microscope (Leica). Propidium iodide (1.5 μM) (Molecular Probes) was used to stain cell nuclei. In all immunohistochemical experiments,

negative controls with pre-immune serum or lacking primary antibody, were included.

RNAi studies

To obtain a dsRNA targeted to *BgVgR* mRNA, a 705 basepair fragment corresponding to the EGF-like domain of BgVgR (from amino acid 660 to 894) was amplified by PCR and subcloned into the pSTBlue^{TM}-1 vector. As control dsRNA, we used a 92 basepair noncoding sequence from the pSTBlue-1 vector (dsControl). Single-stranded sense and antisense RNAs were obtained by transcription *in vitro* using either SP6 or T7 RNA polymerases from the respective plasmids, and resuspended in water. To generate the dsRNAs, equimolar amounts of sense and antisense RNAs were mixed, heated at 95 °C for 10 min, cooled slowly to room temperature and stored at −20 °C until use. Formation of dsRNA was confirmed by running 1 μL of the reaction products in 1% agarose gel. dsRNAs were suspended in diethyl pyrocarbonate-treated water and diluted in Ringer saline. Freshly ecdysed adult females or sixth instar nymphs were injected into the abdominal cavity with a 5 μg dose in a volume of 1 or 0.5 μL, respectively. Controls were injected with the same volume and dose of dsControl.

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