Vitellogenin receptor selectively endocytoses female-specific and highly-expressed hemolymph proteins in the silkworm *Bombyx mori*

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Abstract

Vitellogenin receptor (VgR), a member of the low-density lipoprotein receptor (LDLR) family, functions to transport vitellogenin into the ovaries to promote ovarian growth and embryonic development. In insects, the only widely accepted ligand of VgR is Vg. Recently, *Bombyx mori* VgR (BmVgR) has been shown to interact with *Bombyx mori* storage protein (BmSP1) in vitro. Therefore, in this study, we evaluated whether BmVgR could transport BmSP1 into certain cells. Although BmVgR could combine with *Bombyx mori* Vg (BmVg) and BmSP1, BmVgR did not affect the amount of BmSP1 taken up by Sf9 cells. Parallel immunofluorescence showed that most BmVg and BmVgR were localized in the inner oocyte membrane, showing tissue localization similar to that of BmVg labeled with pHrodo Red absorbed by the ovaries on day 2 of pupation. Although BmSP1 showed localization similar to BmVgR during the same phase, little BmSP1 was present in the ovary. Additionally, BmSP1 did not exist in ovaries when the ovaries contained BmVgR on day 5 of pupation, suggesting that BmSP1 in the ovaries was not endocytosed by BmVgR. In summary, BmVgR could facilitate uptake of BmVg by developing oocytes, but did not modulate in the transport of BmSP1.

Key words: endocytosis; vitellogenin receptor; vitellogenin; storage protein 1; silkworm

42 **Abbreviations**

43 **BmSP1**, storage protein 1 of *Bombyx mori*; **BmVgR**, VgR of *Bombyx mori*; **BmVgR'** , the
44 complete extracellular domain of BmVgR; **BmVg**, vitellogenin of *Bombyx mori*; **EGFP**,
45 epidermal growth factor precursor homology domain; **LBD**, ligand-binding domain; **LDLR**,
46 low-density lipoprotein receptor; **VgR**, vitellogenin receptor; **YPR**, yolk protein receptor.

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61 1. Introduction

62 The vitellogenin receptor (VgR) mediates the endocytosis of vitellogenin (Vg) from the
63 hemolymph to developing oocytes (Sappington and Raikhel 1998; Snigirevskaya et al. 1997).
64 VgR is mainly located at the oocyte membrane surface of almost all oviparous organisms
65 (Goldstein et al. 1985). Disruption of the function of VgR by natural mutations or genetic
66 interference results in the phenotypes of "restricted-ovulator" (Schjeide et al. 1976) and
67 yolkless (Ciudad et al. 2006; Schonbaum et al. 1995).

68 The VgR belongs to the low-density lipoprotein receptor (LDLR) family, and the ligands
69 of LDLR family have been extensively studied. The LDLR family can combine with diverse
70 ligands by employing different combinations of ligand binding repeats (Gliemann 1998;
71 Hussain et al. 1999; Russell et al. 1989). In addition to lipoproteins, proteinases in complex
72 with their inhibitors, carrier proteins for lipophilic molecules, and proteins with specific
73 functions in signaling pathways are known to bind to and enter cells through lipoprotein
74 receptors (Andersen and Petersen 2003). Chicken VgR can also combine with several
75 different exogenous and endogenous ligands (Bujo et al. 1994; Hiesberger et al. 1995;
76 Jacobsen et al. 1995). Although the only reported endogenous ligand of insect VgR is Vg,
77 yolk protein receptor (YPR) from *Drosophila* is structurally very similar to VgRs of other
78 insects; despite sharing these similarities, these proteins recognize quite unrelated ligands
79 (Sappington et al. 1996; Shu et al. 2011; Smith and Reuben Kaufman 2013; Tufail and
80 Takeda 2007; Zhong et al. 2015). Moreover, VgR mRNA is also detected in tissues other than
81 the ovaries, such as the hypopharyngeal glands (Amdam et al. 2003), and fat bodies (Lin et al.
82 2015), suggesting that insect VgRs may also recognize multiple ligands.

83 The domestic silkworm, *Bombyx mori* (Bm) has been used as a Lepidoptera model
84 organism. The full-length cDNA sequence of BmVgR has been obtained and BmVgR has
85 been expressed specifically in the ovaries of silkworm. In silkworm with a partial deletion in
86 the first epidermal growth factor precursor homology domain (EGFP) of BmVgR, the
87 resulting eggs are whiter and smaller than normal eggs, and the homozygote is embryonic
88 lethal (Lin et al. 2013).

89 The silkworm hemolymph circulatory system differs from that of higher animals in that
90 it is an “open” system, bathing the internal organs including the ovaries. As a female-specific
91 protein, BmVg is absorbed by the ovaries during pupation. Another female-specific protein
92 *Bombyx mori* storage protein 1 (BmSP1) (Sakurai et al. 1988), is mainly synthesized in the
93 fat bodies of female silkworms during the larval stage, secreting into the hemolymph and
94 expressed abundantly in the hemolymph on the last day of larval stage. Most BmSP1 is then
95 assimilated by the fat body again. However, few studies have attempted to identify or
96 characterize the BmSP1 receptor. A recent study showed that LBD1 of BmVgR can
97 specifically bind to the BmSP1(Liu et al. 2016).

98 Accordingly, in this study, we used several methods to confirm that the BmVg was
99 endocytosed by BmVgR in silkworms. Additionally, using integrated methods verified in
100 silkworms, we aimed to clarify whether BmVgR could participate in the transport of BmSP1.

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102 **2. Materials and Methods**

103 *2.1. Cell-expression vector construction and expression*

104 We obtained full-length BmVgR from the carrier p50T6kb-9(stored in our laboratory).

The forward and reverse primers for BmVgR (NM_001197251.1) were as follows:
 5'-GAagatctTCATGAAGGTAGTTTTGTTAGCAATAGTTCTATGT-3' and 5'-TTGCGGCCGC
 TTAATTGAGAAATTTATTTTT-3'. BmVgR' is the complete extracellular domain of
 BmVgR and does not affect the interaction with extracellular ligand; thus BmVgR' is a
 secreted protein. The primers for BmVgR' were as follows:
 5'-GAagatctATCAGAATGAAGGTAGTTTTGTTA-3' and
 5'-ATAAGAATgcgccgcTTAAGCGTAGTCTGGGACGTCGTATGGGTAGCAGACACAC
 ACCGGAGC-3'. Polymerase chain reaction (PCR) was carried out at 96 °C for 15 min;
 followed by 5 cycles of 94°C for 40 s, 50°C for 45s, and 72°C for 5min 40 s ; an additional
 30 cycles of 94°C for 40 s, 60°C for 45s and 72°C for 5min40 s and 72°C for 10 min. The
 amplified fragment was cloned into the pMD19-T for sequencing. The primers had BglII and
 NotI restriction enzyme sites, thus the fragment was obtained by digestion with these enzyme
 and then ligated into the psl1180-Hr3-A4-SV40 expression vector (stored in our laboratory).
 The vectors were named as the BmVgR and BmVgR', respectively. Sf9 cells were transfected
 with BmVgR/ BmVgR' using Cellfection II (Invitrogen, Carlsbad, CA, USA). All procedures
 were performed according to the manufacturer's instructions. After refreshing with complete
 medium and culturing the cells for 72 h, membrane proteins from the cells and ovaries were
 extracted using strong RIPA lysis (Beyotime Biotechnology, China), and expression of the
 BmVgR protein was analyzed by western blotting. Transfected cells were also used to
 determine the localization of BmVgR by immunofluorescence assays.

2.2. Insect

Silkworm strain *Dazao* was obtained from the silkworm genebank at Southwest

University, China. All larvae were reared on fresh mulberry leaves, and pupae were placed at room temperature. Hemolymph and ovary samples were dissected from female silkworms at different stages from wandering to day 5 of pupation and were stored at -80°C.

2.3. Purification and fluorescent labeling of BmVg and BmSP1

BmSP1 was purified as described previously (Liu et al. 2016). BmVg was purified using a method similar to that used for BmSP1. The hemolymph on day 3 of pupation was diluted in buffer containing 20 mM Tris-HCl and 100 mM NaCl (pH 7.5) and then centrifuged at $10,000 \times g$ at 4°C for 20 min. BmVg was precipitated as a stable complex using ammonium sulfate at 50–60% saturation and was further purified by Q column, followed by HiLoad Superdex S-200 16/600 column purification (GE Healthcare, UK).

The buffer for pure protein was replaced with 0.1 M freshly prepared NaHCO₃ buffer (pH 8.3) and diluted to a concentration of 1 mg/mL measured using the Bradford method (Bradford 1976) before fluorescently labeling. The stock solution of dye was prepared by dissolving 1 mg pHrodo Red dye (Thermo Fisher Scientific, USA) into 150 µL dimethyl sulfoxide (DMSO). All procedures were performed according to the manufacturer's instructions. The labeled protein was purified by ultrafiltration. The retentate in the ultrafiltration tube of the last ultrafiltrate was the purified BmVg labeled with pHrodo-red, while the permeate was collected and used as a control.

2.4. Co-immunoprecipitation (IP) assay

The culture medium of BmVgR' expressing in Sf9 cells was tested by western blotting with rabbit anti-BmVgR antibodies. A sample of purified BmVg or BmSP1 from silkworm hemolymph was added into the culture medium and incubated at room temperature for 90

min to obtain BmVgR'-BmVg and BmVgR'-BmSP1 complexes. Next, 8.4 µg of rabbit anti-BmVgR antibody diluted in 200 µL of culture medium was added to 50 µL of 5% (w/v) BSA-blocked Dynabeads (Beyotime, China). The Dynabeads were used according to the manufacturer's instructions. In order to exclude interference of non-specifically bound protein, the beads-Ab-BmVgR'-BmVg/BmSP1 complex was washed three times with acidic phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8mM KH₂PO₄ pH=6.5), and the final wash buffer was evaluated. The complex was resuspended in 100 µL of PBS (pH 7.4) and transferred into a clean tube. Next, 20 µL of 5× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added, and the mixture was heated for 10 min at 100 °C. The eluate from the beads was subjected to SDS-PAGE (8% [w/v] polyacrylamide gels) and detected by western-blotting with anti-BmVg and anti-BmSP1 antibody.

2.5. Semi-quantitative reverse transcription (RT-PCR)

Total RNA was extracted from the ovaries of female silkworms using TRIzol (Thermo Fisher Scientific), and cDNA was generated using M-MLV reverse transcriptase (Promega, Madison, WI, USA). All kits were used according to the manufacturer's instructions. The forward primer for *BmSP1* (NM_001113276.2) was 5'-ATTGACTGGCGTAAGGGAG-3', and the reverse primer was 5'-GCTGGGAAGACGGATTTT-3'. PCR was carried out at 94°C for 5 min; followed by 25 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 15 s; and a final extension at 72°C for 10 min. *BmActin3* was used as a control (forward primer: 5'-AACACCCCGTCCTGCTCACTG-3', and reverse primer: 5'-GGGCGAGACGTGTGATTCCT-3'). For *Bmactin*, PCR was carried out at 94°C for 5

min; followed by 25 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 10 min.

2.6. Protein preparation and detection

Protein samples from total ovaries at different phases were prepared as described (Meng et al. 2009). The proteins were subjected to SDS-PAGE (10% [w/v] poly-acrylamide gel) according to the method of Laemmli (Laemmli. 1970). The proteins in the gel were transferred electrophoretically onto polyvinylidene difluoride membranes (Roche, Basel, Switzerland) for western blot assay, and imaged using by the ChemiScope instrument.

Hemolymph specimens were treated as previously described . After separation by SDS-PAGE (10% [w/v] polyacrylamide gels) according to the method of Laemmli, the gel was stained with Coomassie brilliant blue.

2.7. Immunofluorescence histochemistry

On day 2 and 5 of pupation, ovaries were dissected from female silkworms, and immunofluorescence histochemistry was conducted as previously described(Shen et al. 2015) using anti-BmSP1, anti-BmVg and anti-BmVgR antibodies (all antibody were prepared in our laboratory).

2.8. Detection of ligand after incubation with insect cells transfected with BmVgR and ovaries collected on day 2 of pupation

For endocytic uptake, proteins without fluorescent signals were diluted to an appropriate concentration using HEPES buffer, and Sf9 cells were stably transfected in 6 well cell culture plates (for westen blotting) and 24-well cell culture plates (for immunofluorescence), starved for 30 min with Grace's medium without fetal bovine serum, and incubated with a certain

concentration of the ligand protein for 60 min at 27°C after incubating at 4°C for 20 min. The Sf9 cells were then washed with 0.1 M glycine and 0.1 M NaCl (pH 3.0) to remove any uninternalized ligand (Sun et al. 2005). After confirming that BmVgR was expressed in the transfected cells, ligand uptake was detected by immunofluorescence and western blot analysis using anti-BmSP1 and anti-BmVg antibodies. Each uptake experiment was performed three times.

Ovary tissues were excised from pupa on day 2 of pupation and incubated with pHrodo™ red-labeled BmVg at 27°C for 1h. The fluorescence signals of ovaries and ovary ultrathin frozen sections were then observed under a fluorescence microscope (Olympus, Japan).

3. Results

3.1. Expression, localization and in-vitro binding of BmVgR

To clarify the function of BmVgR, we constructed a BmVgR cell-expression vector to analyze the function of BmVgR in Sf9 cells, and another BmVgR' (whole extracellular domain of BmVgR) cell-expression vector to obtain sufficient amounts of protein to perform in-vitro binding assays. The structure schematic of BmVgR and BmVgR' is shown in Fig.1A. BmVgR expressed in Sf9 cells had a molecular weight approximate 250kDa (Fig.1B), consistent with that in the ovary (Fig.1C). BmVgR was located on the outside of the nucleus, i.e., cell membrane and cytoplasm of Sf9 cells (Fig.1D), consistent with its function as a membrane receptor.

LBD1 of BmVgR was shown to interact with BmVg and BmSP1 in a previous study. In our study, the two female-specific and highly expressed protein, BmVg (Fig. 1F) and BmSP1(Fig.1G) were also found to interact with BmVgR in vitro. However, the binding capacity of BmVg was much weaker than that of BmSP1, which may due to acidic buffer (pH=6.5) used to wash the non-specific binding proteins. To the best of our knowledge, the interaction between BmVg and BmVgR was dependent on a neutral pH, while the acidic environment promote dissociation of the interaction (Jeon and Blacklow 2003).

3.2. Uptake of BmVg by Sf9 cells transfected with BmVgR

Next, we performed uptake assays after confirming that BmVgR can be stably expressed in Sf9 cells. We also detected and verified the expression of BmVgR for each endocytosis experiment. After incubating with hemolymph proteins on day 2 of pupation or 1 mg/mL BmVg, Sf9 cells overexpressing BmVgR could assimilate more BmVg (Fig. 2A). A relatively weak band was observed after incubation with 1 mg/mL BmVg, partly because BmVg showed some degradation after storage at a freezing temperature. Subsequently, we re-purified the BmVg protein (Fig. S1-C.D) and confirmed our hypothesis. Immunofluorescence assays further confirmed that BmVg was absorbed into the Sf9 cells (Fig. 2B), avoiding false-positive signal caused by BmVg binding to cell surface protein. Thus, Sf9 cells could be used as the appropriate cells to study the uptake of BmVgR.

3.3. Uptake of BmSP1 by Sf9 cells transfected with BmVgR

BmVgR and BmSP1 can interact with each other in vivo (Liu et al. 2016). Therefore, we next aimed to determine whether BmVgR was involved in the transport of BmSP1 to certain

cells. Because the state of BmVg has an impact on absorption by certain cells, we purified BmSP1 from the hemolymph of female silkworms in the wandering stage(Fig. S1-A.B). Compared with the control, the amount of BmSP1 did not increase in Sf9 cells overexpressing BmVgR (Fig. 3A), and even decrease slightly. Additionally, repeated experiments showed that BmSP1 did not associate with BmVgR entering into cells. Sf9 cells with or without BmVgR could assimilate almost the same amount of BmSP1 into Sf9 cells by immunofluorescence (Fig. 3B). Taken together, these results indicated that BmSP1 entered the cells independent of BmVgR. In other words, BmVgR was not involved in the transport of BmSP1 into Sf9 cells.

3.4. BmVg was transported into the ovaries by BmVgR through co-localization and endocytosis

To determine the relationship between BmVgR and BmVg, ovaries of silkworms on days 2 and 5 of pupation were collected and detected by immunofluorescence. During the early period on day 2 of pupation, BmVgR was distributed near the oocyte membrane and remained evenly distributed in the oocyte cytoplasm (Fig. 4A), whereas BmVg was mainly localized inside the follicular cell layer and oocyte membrane (Fig. 4C) where BmVgR and BmVg could interacted with each other. The BmVgR was mainly located near the oocyte membrane (Fig. 4B) on day 5 of pupation, and the ovaries exhibited the most BmVg in the oocyte cytoplasm (Fig. 4D). These data indicated that BmVgR would return to the oocyte membrane after transporting BmVg into the oocyte cytoplasm.

To further confirm that BmVg was taken up by the ovaries, we used pHrodo red dye to

label BmVg (Fig. S1-E). pHrodo-red was selected because this dye is pH sensitive, the fluorescence intensity is stronger as the pH decreases. To the best of our knowledge, Vg uptake by VgR depends on vesicles, which eventually fuse with the lysosome, making the environment of Vg acidic. As a result, the fluorescence intensity of Vg uptake by the cells was not disrupted by weak interference signals, such as signals from residual dyes in the marked BmVg and extracellular marked BmVg. By SDS-PAGE, the large subunits of BmVg were marked with the red dye. When incubated with the dissociated ovaries on day 2 of pupation, the pHrodo red-labeled BmVg was observed in oocytes and the interspace. Moreover, the BmVg was located in the inner region of the oocyte membrane in ovary slices, similar to the location of BmVg indicated by immunofluorescence on day 2 of pupation (Fig. 4C), supporting that the BmVg was transported into the ovary.

3.5. Expression patterns and localization of *BmSP1* in the ovaries

Next, we aimed to clarify the relationship between BmVgR and BmSP1 in vivo. However, BmVgR was almost undetectable in the fat body when most BmSP1 was absorbed (data not shown). Therefore, we next examined whether BmSP1, which exhibited high levels in the hemolymph, was taken up by the ovaries with high levels of BmVgR in the oocyte membrane. On P2, BmSP1 was localized similar to the BmVgR; however, the BmSP1 was very low (Fig. 5A). A certain amount of BmSP1 protein was detected in female ovaries from wandering to day 3 of pupation (Fig. 5B). RT-PCR showed that the level of *BmSP1* mRNA in the ovary was higher in wandering and very low in pupae (Fig. 5C), with trends similar to those of BmSP1 protein. BmSP1 existed in the hemolymph from wandering to day 5 of pupation (Fig. 5D). On day 5 of pupation, a large amount of BmVgR was expressed in the

ovary and the oocyte membrane (Fig. 4B); however, BmSP1 was not detected in ovary at this phase, suggesting that BmSP1 was completely synthesized in the ovary, or partly absorbed from the hemolymph by an unknown mechanism and receptor. In summary, BmSP1 protein in ovary was also independent of BmVgR.

4. Discussion

Vg is mainly endocytosed by VgR into the ovaries and the endocytosis mechanism has been studied extensively in mosquitos (Snigirevskaya et al. 1997). In our study, BmVgR was distributed in the oocyte cytoplasm and membrane at the early phase of pupation, indicating that BmVgR was synthesized in oocytes and finally located in the oocyte membrane. During this phase, BmVg was localized similar to BmVgR in the oocyte membrane, suggesting that the two proteins have an interacted with each other. During the late phase of pupation, BmVgR was mainly distributed in the oocyte membrane, and BmVg was mainly accumulated in the oocyte cytoplasm, indicating that BmVgR was eventually returned to the oocyte membrane. Similar localization patterns have been observed during vitellogenesis in *Solenopsis invicta* Buren and *Cutthroat trout* (Lu et al. 2009; Mizuta et al. 2013). Vg is synthesized in the female fat body, secreted into the blood and endocytosed by the ovaries (Raikhel and Dhadialla 1992). Moreover, Vg localizes with VgR during the early stage of vitellogenesis and later deposits in the yolk granules. In our study, we acquired similar localization using immunofluorescence and endocytosis of pHrodo red labeled BmVg. Sf9 cells, derived from *Spodoptera frugiperda* ovaries and do not express endogenous BmVgR and BmVg, were selected as the ideal cell. Sf9 cells could absorb more BmVg when it was over-expressed with BmVgR.

300 In silkworms, the uptake of storage proteins by insect fat body cells exhibits several
301 unique features, and it is still unclear whether this process conforms to the standard scheme
302 of eukaryotic endocytosis (Burmester and Scheller 1999). Recently, the receptor responsible
303 for storage protein was identified in *Sarcophaga peregrine* (Ueno and Natori 1984);
304 *Calliphora vicina* (Burmester and Scheller 1997); *Drosophila melanogaster* (Burmester et al.
305 1999); *Corcyra cephalonica* (Damara et al. 2010) and *Helicoverpa zea* (Wang 1994b). In
306 silkworms, the receptor for BmSP1 is still unclear. In contrast, BmVgR is known to bind
307 specifically to another female-specific protein BmSP1 in vitro (Liu et al. 2016). Therefore,
308 we explored whether BmVgR could transport BmSP1 in vivo. Surprisingly, BmVgR was not
309 involved in the transport of BmSP1 into Sf9 cells and silkworm ovaries. The discrepancies
310 between in vitro binding and in vivo uptake may be explained by differences in both
311 conditions, or the physiological effects produced by the interaction of BmVgR and BmSP1
312 may not include endocytosis.

313 Eggs of the BmVgR mutant strain (*vit*) with a deletion of 235-bp in the BmVgR
314 genomic sequence have fewer protein in ovary (Lin et al. 2013), including reduced levels of
315 Vn and 30-kDa proteins. A group of abundant proteins with a molecular weight of ~30 kDa
316 (30-kDa proteins) is synthesized in the silkworm larval fat body and transported into the open
317 circulatory system (hemolymph). These 30-kDa proteins are eventually absorbed by the
318 ovary and exhibit synthesis and transport mechanisms similar to those of BmVg; moreover,
319 these proteins also play an important role in embryogenesis (Pakkianathan et al. 2012; Zhang
320 et al. 2012). Thus, these findings suggest that BmVgR may participate in the transport of
321 30-kDa proteins. Further studies are needed to determine whether these 30-kDa proteins are

transported by BmVgR though a mechanism similar to that of BmVg, as found in this study.

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Competing financial interests

We declare we have no competing interests.

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Figure Legends

Fig.1. Expression, localization and in-vitro binding of BmVgR and BmVgR'. (A) Structural schematic of BmVgR and BmVgR'. S, signal peptide; LBD, ligand-binding domain; EGFP, epidermal growth factor precursor homology domain ; O, O-linked sugar domain; T, transmembrane domain; C, cytoplasmic domain. (B) Proteins extracted from Sf9 cells were detected using anti-BmVgR antibodies. BmVgR and Con indicated the Sf9 cells transfected with BmVgR over-expression and control vector. (C) Proteins of silkworm ovaries on P5 were detected with anti-BmVgR antibodies. (D) The localization of BmVgR in Sf9 cells was detected by immunofluorescence. FITC, localization of BmVgR; MERGE, combined figures of cell nuclei (DAPI) and FITC. (E) Overexpressed of BmVgR' protein in Sf9 cells. (F) BmVgR' interacted with BmVg, as determined by co-IP. (G) BmVgR' interacted with BmSP1, as determined by co-IP.

Fig.2. Uptake of BmVg by Sf9 cells with and without BmVgR. (A) BmVg was absorbed by Sf9 cells after incubating with hemolymph on day 2 of pupation (P2-hem) or 1 mg/mL BmVg (BmVg), and western blotting was used for detection. Tubulin was used as the internal reference. The right figure in (A) shows BmVg protein levels reflected by the relative gray levels of left figure in (A) measured by Image J. Experiments were repeated three times

476 independently, and the relative gray level is shown as the mean $\pm$ SD; ** $P < 0.01$. (B) BmVg
477 taken up by Sf9 cells was detected by immunofluorescence. FITC staining indicated the the
478 localization of BmVg, and the MERGE image showed the combination of FITC and DAPI
479 staining.

480 **Fig.3. Uptake of BmSP1 by Sf9 cells with and without BmVgR.** (A) BmSP1 uptake by Sf9
481 cells was detected by western blotting, and tubulin was used as the internal reference. The last
482 two lanes show the corresponding cell culture medium. The right figure in (B) is the BmSP1
483 protein level, which reflects the relative gray level of the first two lanes in left figure in (A),
484 as measured by Image J. Experiments were repeated three times independently, and the
485 relative gray level is given as the means $\pm$ SD. (B) BmSP1 taken up by Sf9 cells was detected
486 by immunofluorescence. FITC, signal of BmSP1; MERGE image, the combination of FITC
487 and DAPI staining.

488 **Fig.4. Immunofluorescence images of vitellogenin receptor(VgR) and vitellogenin(Vg) in**
489 **silkworm ovaries on P2 and P5, and pHrodo™ red-labeled BmVg uptake by oocyte on**
490 **P2.** (A) The localization of BmVgR on P2. (B) The localization of BmVgR on P5. The white
491 arrows indicate the nuclei of follicular cells, which are localized outside the oocyte, while red
492 arrows indicate the localization of VgR in the oocyte membrane, showing the location of the
493 oocyte membrane. (C) The localization of BmVg on P2. (D) The localization of BmVg on P5.
494 (E) The localization of pHrodo red-labeled BmVg in the ovary and 8- μ m-thick sections of
495 ovary on P2 after incubation with 200 μ g/mL pHrodo red-labeled BmVg. P2, P5; 2, 5 days of
496 pupation. W-ovary, R-ovary, R-ovary-slice, the bright field image of ovaries, figures using the
497 red fluorescence excitation light channel to observe ovaries and ovary-slice. Control and

498 BmVg-Red indicated the permeate and retentate (purified BmVg labeled with phrodo-red) in
499 an ultrafiltrate tube for the last ultrafiltrate, respectively.

500 **Fig.5. Localization and expression patterns of BmSP1 in the ovary.** (A) Localization of
501 BmSP1 in silkworm ovaries on P2 was analyzed by immunofluorescence. (B) BmSP1 protein
502 in the ovaries during different phases. Tubulin was used as internal reference, with 40 µg of
503 total protein in each lane. (C) Gene expression pattern of *BmSP1* in the ovary, as detected by
504 RT-PCR. (D) BmSP1 in the hemolymph was analyzed by SDS-PAGE, with 40 µg total
505 protein in each lane. (L507, day 7 of the fifth instar larvae; W1, W2, and W3: days 1, 2, and 3
506 of wandering; P1, P3, and P5: days 1, 3, and 5 of pupation, respectively).

507 **Fig.S1. Purification and fluorescent labeling of BmVg and BmSP1.** (A) The UV-280 nm
508 curve (indicating the BmSP1 protein content) of the HiLoad Superdex S-200 16/600 column
509 used to purify the BmSP1 protein. (B) The corresponding purified BmSP1 protein collected
510 from the peak on the curve was detected by Coomassie blue staining. (C) The UV-280 nm
511 curve of the HiLoad Superdex S-200 16/600 column used to purify the BmVg protein. (D).
512 The corresponding purified BmVg protein collected from the peak on the curve was detected
513 by Coomassie blue staining. (E) The pHrodo™ red-labeled BmVg protein was detected
514 directly by SDS-PAGE and the subsequent Coomassie blue staining.

Fig. 1

160x180mm (300 x 300 DPI)

Fig. 2

160x180mm (300 x 300 DPI)

Fig. 3

160x180mm (300 x 300 DPI)

Fig. 4

160x180mm (300 x 300 DPI)

Fig.5

160x180mm (300 x 300 DPI)