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Cloning and partial characterization of a gene in *Bombyx mori* homologous to a human adiponectin receptor

Minfeng Zhu, Keping Chen✉, Yong Wang, Zhongjian Guo, Huijuan Yin, Qin Yao and Huiqin Chen

Institute of Life Sciences, Jiangsu University, Zhenjiang, P. R. China

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In this study, we report the cloning and characteristics of an adiponectin-like receptor gene from *Bombyx mori* (BmAdipoR) with highly conserved deduced amino-acid sequences and similar structure to the human adiponectin receptor (AdipoR). Structural analysis of the translated cDNA suggested it encoded a membrane protein with seven transmembrane domains. BmAdipoR was found to be expressed in multiple tissues and highly expressed in Malpighian tubules, fat body and testis. BmNPV (*Bombyx mori* nucleopolyhedrovirus) bacmid system combined with confocal microscopy revealed that BmAdipoR was targeted to the cell membrane. We also found that infection with BmNPV did not have an effect on BmAdipoR mRNA quantity in the midgut of susceptible *Bombyx mori* strain (306) at 48 h, but BmAdipoR mRNA quantity increased significantly at 72 h. We concluded that *BmAdipoR* gene was a membrane protein ubiquitously expressed in *Bombyx mori* tissues and that its expression was altered by treating with BmNPV.

Keywords: BmAdipoR, bioinformatics study, subcellular localization, real-time quantitative PCR, BmNPV

INTRODUCTION

Adiponectin, also termed Acrp30, AdipoQ, apM1 or GBP28, was identified originally by Scherer in 1995 (Scherer *et al.*, 1995), and then by three other groups. It is generally assumed that adiponectin is secreted by the adipose tissue exclusively (Kadowaki *et al.*, 2006). Adiponectin plays a number of beneficial roles in metabolism: improved insulin sensitivity, glucose tolerance and lipid profile (pattern of lipids in the blood), decreased inflammation and atherosclerosis. Its mechanisms of action, active forms, receptors and signaling pathways are not completely defined but seem to involve AMP-activated kinase (AMPK) and downstream acetyl-CoA carboxylase (ACC) (Yamauchi *et al.*, 2002). Adiponectin stimulates glucose utilization and fatty acid oxidation by phosphorylating and activating AMPK (Yamauchi *et al.*, 2002). Activation of AMPK results in phosphorylation of a variety of intracellular proteins and an increase in ATP generation (Winder & Hardie, 1999). In addition, adiponectin increases fatty acid oxidation through inhibition of acetyl-Co A (Co-A) carboxylase and activation of malonyl-CoA decarboxylase resulting in reduced malonyl-CoA content (Tomas *et al.*, 2002). A decrease in malonyl-CoA concentration increases the transport of long chain fatty acyl-CoA molecules into the mitochondria where they are oxidized (Ruderman *et al.*, 1997).

Recently, two adiponectin receptors designated AdipoR1 and AdipoR2 were discovered in Kadowaki's laboratory (Yamauchi *et al.*, 2003). AdipoR1 and R2 belong to a new family of membrane receptors predicted to contain seven transmembrane domains with their N termini as internal parts and their C termini as external parts, which is opposite

✉Corresponding author: Keping Chen, Institute of Life Sciences, Jiangsu University, #301 Xuefu Road, Zhenjiang 212013, P. R. China; tel: 86-511-8879 1923; fax: 86-511-8879 1923; e-mail: kpchen@ujs.edu.cn

Abbreviations: AdipoR, adiponectin receptor; AMPK, AMP-activated kinase; ACC, acetyl-CoA carboxylase; ARH, arcuate hypothalamus; BmAdipoR, *Bombyx mori* adiponectin receptor; BmNPV, *Bombyx mori* nucleopolyhedrovirus; EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; GPCRs, G protein-coupled receptors; hpi, hours post infection; MCS, multiple cloning sites; MOI, multiplicity of infection; TM, transmembrane.

to the topology of all other reported G protein-coupled receptors (GPCRs). These proteins are structurally conserved from yeast to man (especially in the seven transmembrane domains) (Yamauchi *et al.*, 2003). Interestingly, the AdipoR1 yeast homolog YOL002c plays a key role in metabolic pathways that regulate lipid metabolism such as fatty-acid oxidation (Karpichev *et al.*, 2002). In humans and rodents, AdipoR1 and AdipoR2 are expressed ubiquitously. While AdipoR1 is abundantly expressed in skeletal muscle, AdipoR2 is predominantly expressed in the liver (Yamauchi *et al.*, 2003). Both *AdipoR1* and *AdipoR2* genes are expressed in human monocytes and macrophages (Chinetti *et al.*, 2004) and also in a primary human osteoblast cell line (Berner *et al.*, 2004) and arcuate hypothalamus (ARH) (Kadowaki *et al.*, 2008). In addition, *AdipoR1* and *AdipoR2* genes are expressed in rat and human pancreatic β cells at levels similar to those expressed in the liver, but greater than that in muscle (Kharroubi *et al.*, 2003). The expression of AdipoR1 and/or AdipoR2 is regulated under physiological and pathophysiological states including fasting/refeeding, obesity and insulin resistance (Tsuchida *et al.*, 2004).

BmNPV is a nuclear polyhedrosis virus (NPV) that has caused the highest damage to *Bombyx mori* in tropical regions in recent years. BmNPV affects midgut epithelial cells, trachea system, hemolymph cells, fat body, etc. The nuclear of middle and inner cells of the silk gland are also sometimes invaded by this virus (Khurad *et al.*, 2004). The processes of BmNPV infection cause many biochemical changes in larvae which respond to these biological phenomena by many changes of their metabolism to defend against pathogen invasion.

In the present study, we cloned a *Bombyx mori* adiponectin-like receptor gene which is closely related to human AdipoR and we provide novel evidence that BmAdipoR is expressed in multiple tissues including Malpighian tubules and silk glands. We also provide evidence that expression of the gene encoding BmAdipoR in the midgut of silkworm species 306 is increased significantly at 72 h by treatment with BmNPV.

MATERIALS AND METHODS

Animals. The highly susceptible silkworm strain 306 (LC50 = 4.95 BmNPV polyhedra/ml) was raised in our lab (Xu *et al.*, 2005). All larvae were raised with mulberry up to the fifth instar. Fifty newly exuviated fifth instar larvae were selected and fed with mulberry treated with 3.6×10^8 BmNPV polyhedra/ml (BmNPV T3 strain).

Identification of the putative BmAdipoR. **H**uman AdipoR2 (GenBank Accession No. NP_078827) sequence was used to conduct TBLASTN searches against silkworm EST database using NCBI default parameters. The obtained EST sequence with the highest identity was used to perform additional iterative BLASTN searches to obtain silkworm EST sequences for gene assembly. The cDNA sequence of BmAdipoR was obtained by EST assembly using SeqMan program of DNAstar.

Structural and phylogenetic analysis. Computer analyses (PREDICT PROTEIN, TMPRED, and TOPPRED (Claros & von Heijne, 1994)) were used to predict the likely structure of the protein and its localization within the cell based on the deduced amino-acid sequence. The multiple protein sequence alignments were carried out using the program Clustal X and the parameters were auto-generated. The molecular evolutionary genetic tree through the Neighbor-joining method was constructed using MEGA3 (Kumar *et al.*, 2004). The phylogenetic tree was tested using Bootstrap (1000 replicates; seed = 64,238). Pairwise deletion was selected for gaps/missing data.

RT-PCR. Total RNA was extracted from silkworm (the fifth instar larvae) fat body, midgut, ovary, testis, Malpighian tubules, blood and silk gland using RNeasy kit. Following DNase-I treatment, first strand cDNA was synthesized by reverse transcription of 2 μg of total RNA using $d(T)_{30}$A/G/CA/G/C/T primer and 2U reverse transcriptase. Approximately 100 ng of single stranded cDNA was used as template to amplify a 1116 bp product of BmAdipoR, using the following primers (forward: 5′-A**TCTAGA**ATGGATTGCGACGCAGGCA-3′; reverse: 5′-**CTCGAG**AAATGCCATTGATGTTGGTG-3′) which synthesized the predicted sequences with *Xba*I and *Xho*I sites (shown in bold), respectively. RT-PCR was performed using the following program: 94°C for 90 s, 36 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 2 min. The PCR products were subject to agarose gel electrophoresis and ethidium bromide staining for visualization. A Bm-actin A3 gene fragment was also amplified as a positive control, with primers actin F (5′-GGATGTCCACGTCGCACTTCA-3′) and actin R (5′-GCGCGGCTACTCGTTCACTACC-3′). The RT-PCR product from each tissue was sequenced to confirm authenticity and used to construct recombination virus vBm-BmAdipoR-EGFP.

The generation of recombinant virus vBm-BmAdipoR-EGFP and expression of EGFP-BmAdipoR fusion protein in BmN-4 cells. For the BmAdipoR gene, the pFas Bac HTb baculovirus transfer vector was used. The genes obtained above were inserted between *Xba*I and *Xho*I sites in the multiple cloning sites (MCS). The plasmid pFasBacHTb-BmAdipoR-EGFP was used to transform competent

DH10B cells containing helper and BmNPV bacmid. The transformed cells were added to 800 μl of SOC and incubated at 37°C for 4 h, and then were cultured on Luria-Bertani (LB) agar plates containing gentamicin, kanamycin, tetracycline, IPTG, and X-Gal at 37°C overnight. After two rounds of color selection, white colonies containing *BmAdipoR-EGFP* gene were obtained and inoculated into 3 ml of LB medium. The presence of *BmAdipoR-EGFP* gene in the recombinant bacmid was verified by PCR. BmN-4 cells were transfected with recombinant bacmid (approx. 200 ng) using Cellfectin Reagent (Gibco-BRL) to produce the recombinant baculovirus vBm-BmAdipoR-EGFP.

BmN-4 cells were infected with vBm-BmAdipoR-EGFP at a multiplicity of infection (MOI) of 5. To test the subcellular localization of BmAdipoR, we used the Olympus Laser scanning confocal microscopy system, and identified mitochondria and nuclei by staining with Mito Tracker Red CMXRos and Hoechst 33258, respectively.

BmAdipoR mRNA quantitation in various tissues by real-time quantitative PCR. Total RNA was extracted from the silkworm fat body, midgut, ovary, testis, Malpighian tubules, blood and silk gland as described above. One microgram of total RNA was reverse transcribed using $d(T)_{30}$A/G/C A/G/C/T primer and 2 U reverse transcriptase in a 20 μl reaction system. BmAdipoR mRNA and Bm-actin A3 mRNA were quantified utilizing 2 μl of the reverse transcription reaction (equivalent to 100 ng single-stranded cDNA) as template in the real-time quantitative PCR. A 198-bp product for BmAdipoR cDNA corresponding to nucleotides 68–265 (GenBank Accession no. EF062308) was amplified using the following primers: forward 5′-CGACGAAGTCCTCGCAGAA-3′ and reverse 5′-TCCACGACGCCTCCCATAC-3′. Similarly, a 284 bp product of silkworm Bm-actin A3 was amplified using the following primer set: forward 5′-GGATGTCCACGTCGCACTTCA-3′ and reverse 5′-GCGCGGCTACTCGTTCACTACC-3′. The real-time quantitative PCR was performed on MX3000PTM Real-time PCR System. The thermal cycling profile consisted of initial denaturation at 95°C for 3 min and 40 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s. The relative expression level was analyzed with $2^{-\Delta\Delta}C^{T}$ method.

Effect of treatment with BmNPV on BmAdipoR mRNA expression. The 5th instar larvae of silkworm were treated with BmNPV at 24 hpi, 48 hpi and 72 hpi. Midguts were collected and frozen in liquid nitrogen and stored under −70°C until analyzed. Total RNA was extracted using RNeasy kit. One microgram of DNase-I treated total RNA was reverse transcribed as described above. BmAdipoR mRNA and Bm-actin A3 mRNA quantities were determined by real-time quantitative RT-PCR using SYBR® Green dye as described above. The amount of AdipoR mRNA was expressed as a proportion to Bm-actin A3 mRNA as described above and compared between 306 and NB.

RESULTS

Identification of the full-length cDNA sequences

TBLASTN searches against silkworm EST database with human AdipoR2 revealed one EST fragment (GenBank Accession No. CN211900) whose deduced amino-acid sequence has obvious sequence identity with it. After iterative BLAST searches and EST assemblies, we obtained the full-length cDNA sequence of BmAdipoR. Its putative amino acid sequence contained a HlyIII motif which was identical to that of human AdipoR2 (Fig. 1).

Phylogenetic and structural analyses of the BmAdipoR

AdipoR proteins are characterized by a 7 TM pass organization wholly contained within the UPF0073 motif. Comparison of the amino-acid sequence between species revealed that the predicted transmembrane regions are highly conserved especially in the TM3 (Fig. 2). Regions external to the TM domains are predicted to form intra- or extracellular loops. This shows that the invariant residues are found predominantly in the intracellular loops (Fig. 2). Outside of the TM 7 domain, the AdipoR proteins have distal N- and C-terminal regions. The N-terminal regions have various lengths, with highly conserved sequences near the TM domain, while the C-terminal regions have similar lengths, with relatively low sequence homologies (Fig. 3). However, a clear pattern of invariant residues ($HGXSX_5RX_6C$) within the C-terminal regions can be seen in this alignment (Fig. 2). The results of the phylogenetic analysis indicated that four major clades of AdipoRs, vertebrate, invertebrate (insects), invertebrate (*Nematode*), and *Fungi*, exist in vertebrate and invertebrate classes (Fig. 4). The BmAdipoR is classified into the invertebrate (insects) subgroup. Sequence identity comparisons also showed that BmAdipoR had a higher homology with the invertebrate (insects) group compared with other groups (not shown).

A proposed model for the insertion of the putative BmAdipoR in the plasma membrane based on hydrophobicity and charges of the amino acids analyzed by TopPred (Claros & von Heijne, 1994) is shown in Fig. 5. It is shown that the N-terminal ends of BmAdipoR are localized intracellularly while their

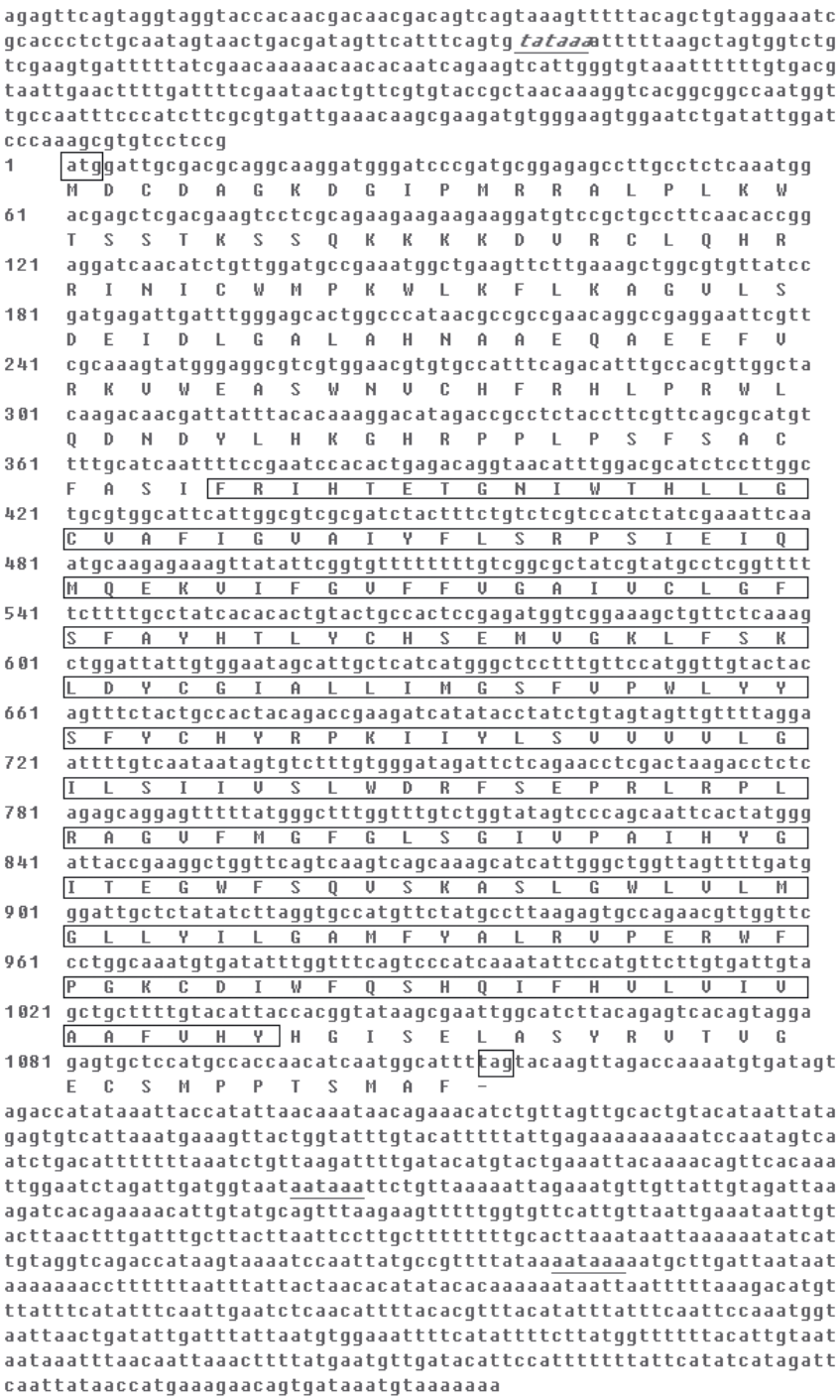

Figure 1. Nucleotide sequence and deduced amino-acid sequence of the BmAdipoR.
The predicted amino acid is represented by the one letter code designation below the nucleotide sequence. The TATA-box is in italics and underlined. PolyA signals are underlined. In the amino-acid sequence, the conservative amino-acids (HlyIII) are framed.

C-terminal ends reside in the extracellular domain, which is in contrast to the GPCRs model.

Expression of BmAdipoR in various tissues

RT-PCR analysis revealed that BmAdipoR mRNAs were distributed in a wide range of *Bombyx mori* tissues (Fig. 6). Real-time quantitative PCR analysis showed that BmAdipoR mRNA was the highest in Malpighian tubules, fat body and testis, followed by ovary, blood, silk gland and midgut (Fig. 6). Melting curve analyses showed the presence of a single PCR product for BmAdipoR or Bm-actin A3, confirming the specificity of the reaction (not shown).

Expression of EGFP-BmAdipoR fusion protein in BmN-4 cells

Previous studies revealed that AdipoR is a membrane protein (Yamauchi *et al.*, 2003). To characterize BmAdipoR, the gene was inserted into an ex-

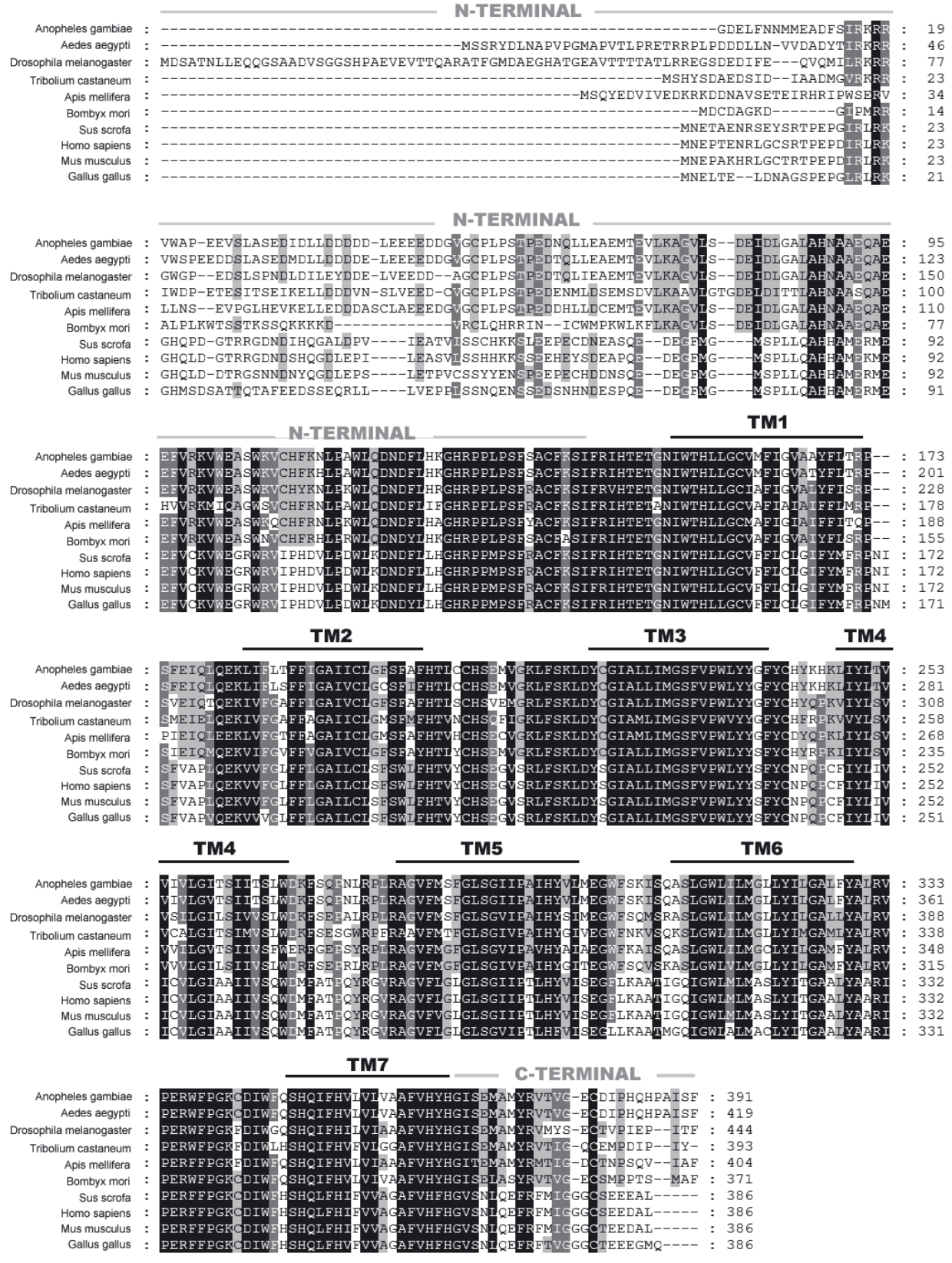

Figure 2. The multiple sequence alignments of BmAdipoR with those of other species.
Numbers on the right indicate the amino-acid position of different sequences. Identical amino acids are shaded in black. Black bars over amino-acid residues indicate the seven transmembrane domain regions as predicted by TMPRED. Gray bars over amino-acid residues indicate the distal N- and C-terminal regions.

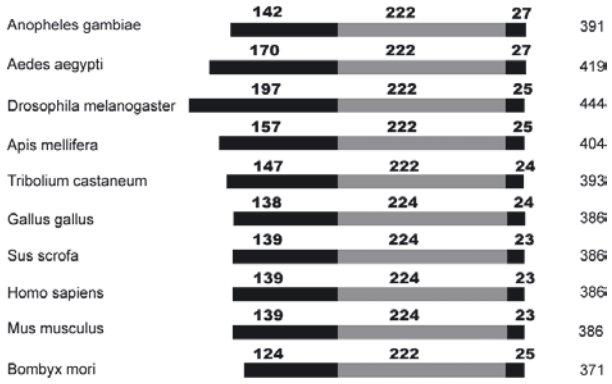

Figure 3. Relative sizes of AdipoR in different species are indicated.
Each AdipoR protein encodes a predicted intracellular N-terminal domain, a 7TM spanning sequence (encoded within the UPF0073 Pfam domain), and a predicted extracellular C-terminal domain.

pression vector that allows the production of EGFP or BmAdipoR-EGFP fusion protein. vBm-BmAdipoR-EGFP plasmids were transfected into BmN-4 cells. Localization of BmAdipoR protein was tested by fluorescence confocal microscopy. It was shown that EGFP protein was distributed throughout the entire cell in both cytoplasm and nucleus. However, BmAdipoR-EGFP was located in the cell membrane (Fig. 7).

Effect of treatment with BmNPV on BmAdipoR mRNA quantity

BmAdipoR mRNA quantity did not change significantly at 48 h in strain 306 midgut infected with BmNPV, but increased significantly at 72 h (Fig. 8). Melting curve analyses showed the presence of a single PCR product for BmAdipoR or Bm-actin A3, confirming the specificity of the reaction (not shown).

DISCUSSION

We have cloned and partly characterized a new adiponectin-like receptor in *Bombyx mori* that shares significant sequence identity with human AdipoR. By comparing BmAdipoR with AdipoRs in other animals, we found that BmAdipoR has highly conserved transmembrane regions. Structure analysis showed that the invariant residues are found predominantly in the intracellular loops or the in-

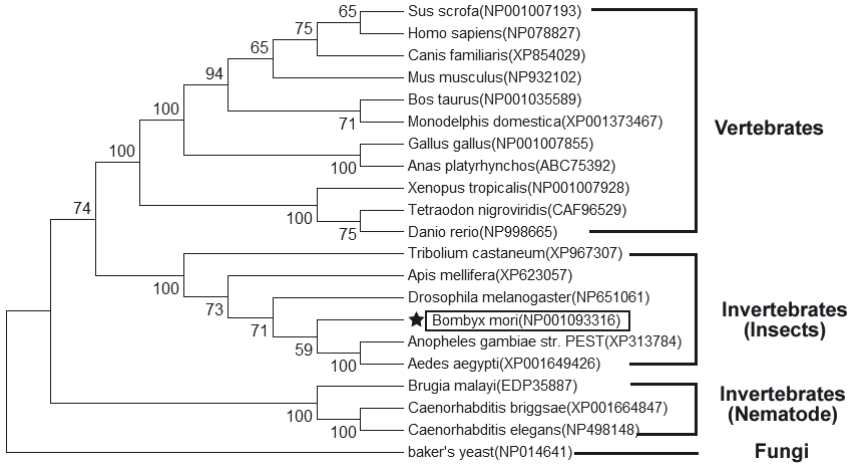

Figure 4. Phylogenetic analysis of BmAdipoR with other AdipoR family members.
NJ tree was produced by MEGA3 which shows the four indicated clades of AdipoR. One thousand bootstraps were performed for checking reproducibility of the result.

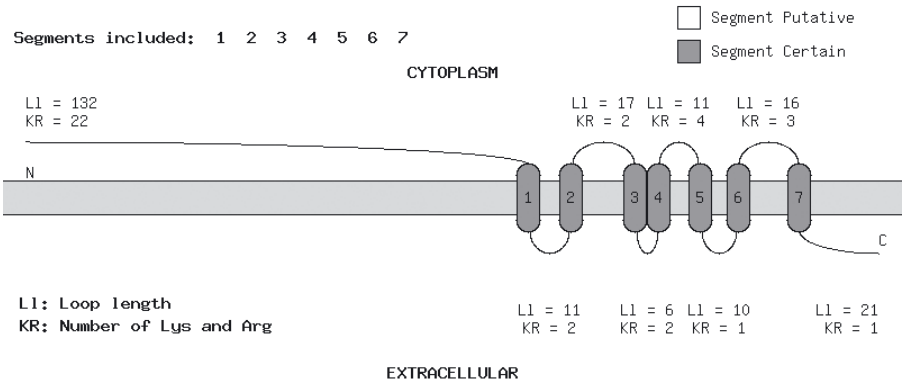

Figure 5. Topological model for BmAdipoR protein.
Transmembrane spanning regions (Segments) were predicted using TopPred (Claros & von Heijne, 1994). Loop length and number of Lys and Arg are indicated.

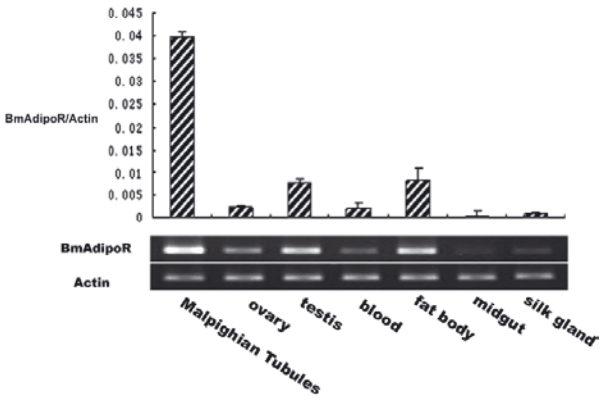

Figure 6. The relative levels of BmAdipoR from various tissues of *Bombyx mori* were measured by semi-quantitative RT-PCR and real-time quantitative PCR.
Using either method, the highest concentration of BmAdipoR was found in Malpighian tubules.

tracellular face of the TM domains, indicating that conserved features may be required for signal transduction and that the situation may be similar to that observed in GPCRs; for example, the rhodopsin-type GPCRs have relatively low sequence similarities, yet they maintain a 7TM architecture with invariant residues observed within the intracellular loops and much less conservation in the extracellular loops (Gether, 2000). The conserved sequences of N-terminal regions and the invariant residues (HGX-SX_5RX_6C) within the C-terminal regions may be the ligand binding sites. Phylogenetic comparisons of the deduced amino-acid sequence of BmAdipoR with homologous sequences from a variety of other species showed that BmAdipoR is clustered in the subgroup of invertebrates (insects) (Fig. 4). Computer analysis using a program that predicts protein structure (TopPred) indicates that the deduced protein is located in the plasma membrane and typically has seven transmembrane domains as well as intracellular N-terminal and extracellular C-terminal domains. This protein structure is similar to those of mammalian AdipoR1 and AdipoR2 which are opposite to the topology of all other reported GPCRs.

RT-PCR showed that *BmAdipoR* was expressed in multiple tissues in *Bombyx mori*, this pattern was similar to that of mammalian AdipoR that is expressed ubiquitously. We determined the relative expression of BmAdipoR mRNA in various tissues using real-time quantitative PCR and found that Malpighian Tubules, fat body and **testis** were the principal organs where *BmAdipoR* gene was maximally expressed. In most insects the fat body serves as a storage depot for food reserves. In addition to its important roles as a storage depot, the fat body of insects functions as a key center of metabolism and biochemistry. The BmAdipoR highly expressed in *Bombyx mori* fat body may possibly be involved in lipid metabolism and glucose utilization. It is tempting to speculate that BmAdipoR mRNA expressed in the testis was possibly involved in steroid synthesis as it was recently shown that AMPK, a molecule that is activated by adiponectin, is involved in progesterone synthesis

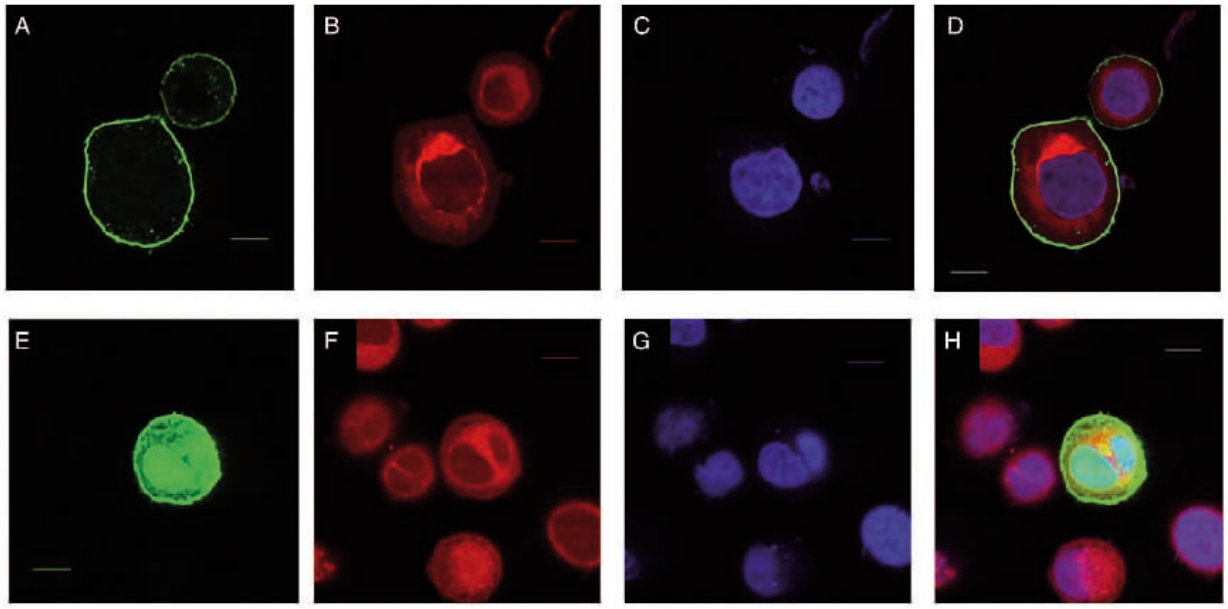

Figure 7. Cellular localization of BmAdipoR within transfected cells.
A. The cells transfected with vBm-BmAdipoR-EGFP plasmids; E. The cells transfected with vBm-EGFP plasmids; B and F. Mitochondria were stained red with Mito Tracker Red CMXRos; C and G. Nuclei were stained blue with Hoechst 33258; D. Merged images of panel A, B, and C; H. Merged images of panel E, F, and G. Bar = 10μm.

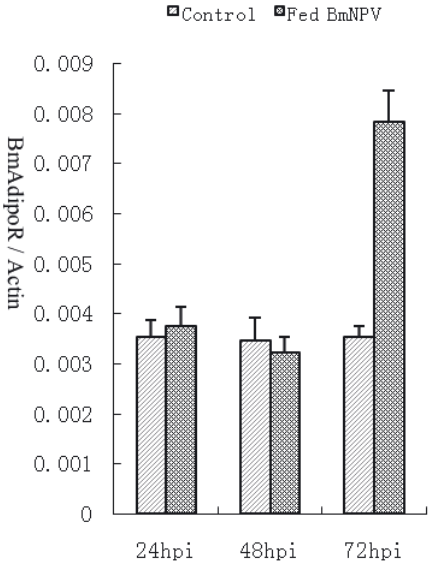

Figure 8. Effect of feed BmNPV on BmAdipoR mRNA quantity in the 306 midgut.
The 5th instar silkworm larva was reared on fresh mulberry leaves without any treatment (control). Treatment group were fed with mulberry treated with 3.6×10^8 BmNPV polyhedra/ml (BmNPV T3 strain). hpi: hours post-infection.

(Tosca *et al*., 2005), and the rapid actions of progestins can increase sperm motility and fertilization (Thomas *et al*., 2005).

This is the first report describing robust expression of AdipoR mRNA in the Malpighian tubules. Good osmoregulation is critical to the success of insects, and the Malpighian tubules play a key role in osmoregulation. Recently, the application of genetics and genomics to the *Drosophila* tubule has revealed far more extensive roles than ion and water transport. Microarray analysis shows that organic solute transporters dominate the tubule transcriptome (Wang *et al*., 2004). The tubule thus has the capability to excrete actively a broad range of organic solutes and xenobiotics. Such transporters can produce unexpected, emergent roles for the whole tissue. Reinforcing this role in excretion, the tubule expresses very high levels of a particular cytochrome P450 s, glutathione-*S*-transferases and alcohol dehydrogenases, which suggests that the tubule plays a major role in metabolism and detoxification of both endogenous solutes and xenobiotics, such as insecticides (Dow & Davies, 2006). The function of BmAdipoR in *Bombyx mori* Malpighian tubules is not clear. It may be involved in lipid metabolism of organic solutes, making toxic compounds less harmful, or at least easier to excrete.

Subcellular localization of proteins is particularly helpful in the functional annotation of gene products. In this study, we used the BmNPV bacmid system to examine the subcellular localization of BmAdipoR. The result showed that BmAdipoR was a membrane protein, which is consistent with previous studies (Yamauchi *et al*., 2003). This further confirmed that BmAdipoR may have the same function as mammalian AdipoR.

To see whether the BmAdipoR was involved in BmNPV infection, we investigated the effect of BmNPV infection on the expression of BmAdipoR mRNA in *Bombyx mori* midguts. In the present study, we found that BmAdipoR mRNA quantity did not change in strain 306 infected with BmNPV at 48 h, but increased significantly at 72 h. Previous studies showed that infection with BmNPV did not have a considerable effect on carbohydrates up to the third day but with time, the amount of carbohydrates was enhanced in infected *Bombyx mori* (Rami reddy *et al*., 1992). A significant increase in BmAdipoR mRNA quantity at 72 h in response to treatment with BmNPV may enhance the ability of adiponectin to stimulate glucose uptake. In this regard, the increased carbohydrates in larvae treated with BmNPV were at least partly caused by the increased BmAdipoR. Nevertheless, the reasons for the significant change in BmAdipoR mRNA quantity in response to treatment with BmNPV require further studies.

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