Research Article

Cloning and functional characterization of a vertebrate low-density lipoprotein receptor homolog from eri silkmoth, *Samia ricini*

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Abstract

The lipophorin receptor (LpR) is the insect lipoprotein receptor and belongs to the low-density lipoprotein receptor (LDLR) superfamily. It has a vital role in the uptake of lipophorin (Lp) into various tissues. Here we report the full length cloning and functional characterization of an LpR from eri silkmoth, *Samia ricini*. The full length cDNA of *Sr*LpR7-1 is 4132 bp including an open reading frame (ORF) of 2595 bp. The deduced amino acid sequence revealed well structured ligand binding, epidermal growth factor, glycosylation, transmembrane and cytoplasmic domains. The ligand binding domain consisted of seven cysteine repeats instead of the common eight cysteine repeats indicating it as a homolog of human LDLR. We identified another splice variant, *Sr*LpR7-2 with a deletion of 27 amino acids in the *O*-glycosylation domain. Apart from the fat body, both isoforms are expressed in ovary, brain and other tissues at different developmental stages of the silkworm. RNAi experiments did not show any marked effects except that the adult emergence was delayed compared to controls. In addition, the *Sr*LpR7 cDNA was recombinantly expressed and ligand binding experiments confirmed that the receptor protein binds not only to *Sr*Lp but also to *Bombyx mori* Lp.

Introduction

Lipophorin (Lp) is critical in insects for lipid transport between tissues. The loading and unloading of lipids into and from fat body is accomplished by Lp and another protein called lipid transfer particle (LTP) (Arrese *et al*. 2001, Ryan & Van der Horst 2000). Lp functions as a reusable lipid shuttle and the delivery of lipids to various organs occurs without its internalization, accumulation or degradation (Van der Horst *et al*. 2002). Based on its density, Lp is classified as low, high and very high-density. Lp incorporates into the cells through receptor-mediated endocytosis or without endocytosis (Parra-Peralbo & Culi 2011, Raikhel & Dhadialla 1992, Rodenburg & Van der Horst 2005, Van Hoof *et al*. 2003). During lepidopteran vitellogenesis, Lp transports lipids and other yolk precursors from the fat body to the ovaries and, in some species, Lp itself becomes one of the major constituents of the protein yolk bodies (Sun *et al*. 2000, Swevers *et al*. 2005, Telfer *et al*. 1991).

The Lipophorin receptor (LpR) belongs to the low-density lipoprotein receptor (LDLR) superfamily. The members of this family bind to various ligands and are involved in the lipid metabolism of both vertebrates and invertebrates (Schneider & Nimpf 2003). The defining structural features of the family consist of i) a cysteine-rich ligand binding domain (LBD), ii) an epidermal growth factor domain (EGFD), iii) an *O*-linked sugar domain (OSD), iv) a transmembrane domain (TMD), and v) a cytoplasmic domain (CD). The number of cysteine-rich repeats in LBD varies among LDLR family members. LDLR contains seven cysteine -rich repeats whereas VLDLR has eight such repeats and large receptors like megalin and LDLR-related proteins have more cysteine-repeats in their LBDs (Willnow *et al*. 1994). LpRs have been cloned and characterized from *Locusta migratoria* (Dantuma *et al*. 1999), *Aedes aegypti* (Cheon *et al*. 2001, Seo *et al*. 2003), *Galleria mellonella* (Lee *et al*. 2003), *Bombyx mori* (Gopalapillai *et al*. 2006), *Blattella germanica* (Ciudad *et al*. 2007), *Apis mellifera* (Guiugli-Lazzarini *et al*. 2008) and *Leucophaea maderae* (Tufail *et al*. 2009). Most of these LpRs are homologues to

VLDLR, having eight cysteine-rich repeats in their LBDs. However, *Aedes aegypti* (fat body) (Seo *et al*. 2003), *Blattella germanica* (Ciudad *et al*. 2007) and *Drosophila melanogaster* (Parra-Peralbo & Culi 2011) LpRs have seven cysteine rich-repeats in their LBDs and thus resemble human LDLR.

Samia ricini (Lepidoptera: Saturniidae), the Indian eri silkworm, contributes significantly to the production of commercial silk (known for its white or brick-red eri silk) and is the only domesticated non-mulberry silkworm. It is distributed in India, Japan and China. The eri silk is also used for studies in biomedical applications of silk proteins (Pal *et al*. 2013). The Lp of the *S. ricini* is a high density lipoprotein composed of two apolipophorin subunits with a molecular mass of approximately 260 (Apo-I) and 80 kDa (Apo-II), respectively (manuscript submitted). As noted above, several LpR genes have been cloned and characterized in recent years including our work on *B. mori*. However, knowledge on the functional aspects of LpR and its interactions remain limited. The present report describes the primary structure of the *Sr*LpR gene, tissue and stage-specific expression patterns, recombinant protein expression and functional characterization. Additionally, apart from showing variations in RNAi efficiency, the cross reactivity of *Sr*LpR with *Bm*Lp is also demonstrated.

Materials and Methods

Cloning of *Sr*LpR cDNA

The eri silkworms, *S. ricini* were maintained in our laboratory and fed on castor leaves. Total RNA was purified from the fat body of Day 10 pupae using RNeasy mini kit (Qiagen). Reverse transcription was performed using oligo (dT) with Primescript RT-PCR system (Takara). Two degenerative primers (Forward: 5'GTITAYTGGACIGAYTGGGAYAA-3'; Reverse: 5'- TAIACIGGRTTRTCRAARTTCAT-3'; I-inosine) designed from the consensus sequences of LpRs were used to amplify a partial sequence of *Sr*LpR. The PCR product was cloned and sequenced. The 5' and 3' RACE kit (Roche) was used to obtain the full length *Sr*LpR cDNA using gene specific primers (SP1-5: 5'-CAGCATCGCGGCCACTGTTTGAGA-3'; SP2-5: 5'-CGCACATCTGGTTGTCGGGGAGT-3'; SP3-5: 5'-TCCTGGGTGCAGGTAAGCAGAGGT-3'; SP5-3: 5'-ACTCCCCGACAACCAGATGTGCG-3'; SP6-3: 5'-TCT CAAACAGTGGCCGCGATGCTG-3') and adapter primers from the kit according to the manufacturer's instructions. The amplified products were cloned and sequenced. Several independent clones were used to eliminate possible PCR errors. Full-length cDNA was obtained by PCR using high-fidelity Accuprime DNA polymerase (Invitrogen).

RT-PCR

Tissues from fifth instar larvae, pupae (Day 12) and adult moths (Day 1) were dissected and RNA was extracted as above. Equal amount of RNA was used for reverse transcription. First-strand cDNA was synthesized using Primescript RT-PCR system (Takara) and was used as template for PCR. The primers employed for the *Sr*LpR-1 and *Sr*LpR-2 were: Forward: 5'-ATACCAGAGGAGGTGCATATAACG-3' and Reverse: 5'-CGATGTAACATTATGGTGAACGT-3'; Forward: 5'-ATTCGCCCCGCGTCTCCTGCGCTTG-3' and Reverse: 5'-CGATGTAACATTATGGTGA-ACGT-3', respectively. The forward primer for the *Sr*LpR7-1 expression was designed from the glycosylation domain having an addition of 81 nucleotides, the only divergent region specific to *Sr*LpR7-1. However, for the detection of *Sr*LpR7-2, primers were designed from a region common to both isoforms. The 18S rRNA was used as an internal control (Forward: 5'-TTGACGGAAGGGCACCACCAG-3' and Reverse: 5'- AGACGCCGGTCCCTCTAAGAAG-3'). Random hexamers were used for reverse transcription for 18S rRNA amplification. Care was taken to avoid genomic DNA contamination. Each RT-PCR product was verified by sequencing.

RNA interference (RNAi)

To obtain dsRNA targeted to both LpR mRNAs, a fragment was amplified by PCR using T7 promoter and gene-specific primers (Forward: 5'-TAATACGACTCACTATAGGGAGAACGTGCCA-GCCGCAGTCACATTTCG-3'; Reverse: 5'-TCTCCCTATAGTGAGTCGTATTAACAACGTGT-ACGGTCGCGTGGATCAC-3') and GFP as control (Forward: 5'-TAATACGACTCACTATAGGGAGA GCTACCTGTTCCATGGCCAACACTTG- 3'; Reverse: 5'- TCTCCCTATAGTGAGTCGTAT-TATGTCTGGTAAAAGGACAGGGCCATCGC-3'). The GFP plasmid was kindly given by Professor K. P. Gopinathan, Indian Institute of Science, Bangalore, India. The strategy employed was a single PCR with T7 promoter appended to 5' ends of both forward and reverse PCR primers as indicted above, to generate transcription template for both strands of the dsRNA. One μg of each PCR product was subjected to *in vitro* transcription. The *in vitro* transcriptions were carried out using MEGAscript RNAi Kit (Ambion) following the manufacturer's instructions except that the reaction mixes were incubated for 6 h at 37°C to increase dsRNA yield. This was followed by nuclease digestion to remove DNA and ssRNA, and purification of

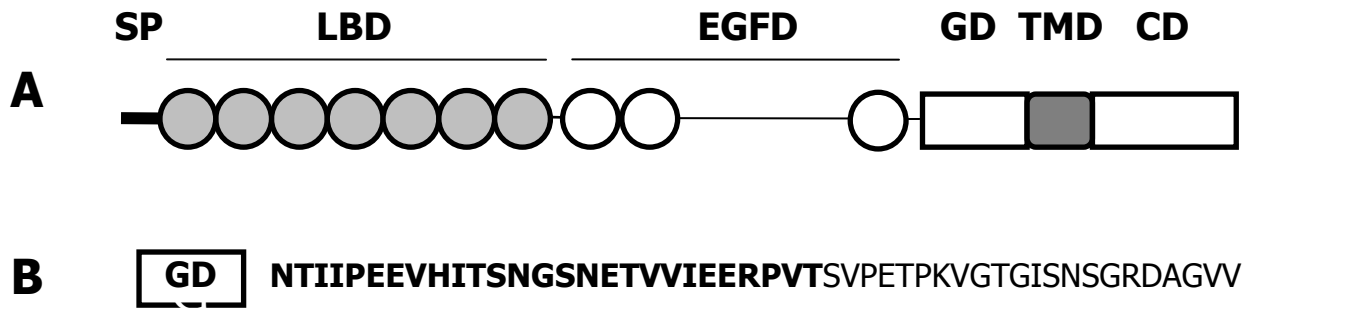

Figure 1. A: *Sr*LpR with seven cysteine repeats. SP: Signal peptide; LBD: Ligand binding domain; EGFD: Epidermal growth factor domain; GD: Glycosylation domain; TMD: Transmembrane domain; CD: Cytoplasmic domain. B: Addition of 27 amino acids (in bold) in GD.

dsRNA was carried out according to manufacturer's instructions. The quantity and integrity of dsRNA were analysed using Nanodrop 2000 (Thermo Fisher Scientific) and agarose gel electrophoresis. Female pupae (Day 1) of *S. ricini* were injected with 50 µg of dsRNA, into intersegmental membrane between 8th and 9th abdominal segments. Pupae were again injected with the same dose on Day 10. Water injected pupae and normal females without any injection were also used as negative controls. RT-PCR was used to investigate the expression of LpR mRNA in ovaries of Day 12 pupae using the above gene-specific primers. The female moths were mated with normal males in all groups. Observations on ovarian development, adult emergence, fecundity and hatching were recorded.

Isolation of lipophorin (Lp) and preparation of antibodies

Hemolymph from mid fifth instar female larvae (non-vitellogenic) of *S. ricini and B. mori* were collected, hemocytes were removed, and Lp was collected by KBr density gradient ultracentrifugation (Shapiro *et al*. 1984, Ravikumar *et al*. 2011). HDLp which formed a clear yellow band was collected, desalted, and used immediately for the binding assays. The density was 1.103 g/ml for *Sr*Lp and 1.064 g/ml for *Bm*Lp. The purified *Sr*Lp was confirmed by 5 % SDS-PAGE and polyclonal antibodies (Apo I and II) were produced in rabbits by Bhat Biotech India Pvt Limited, Bangalore, India. The *B. mori* HDLp antibody was kindly given by Dr. Kozo Tsuchida, National Institute of Infectious Diseases, Tokyo, Japan.

Protein expression and ligand binding

The cDNA of *Sr*LpR7-1 (without signal sequence) and with polyhistidine tag was cloned into pF25A ICE T7 Flexi Vector (Promega) between *SgfI* and *PmeI* sites. After sequence verification, the constructed plasmid was used for *in vitro* transcription coupled translation using TNT-T7 Insect Cell Extract Protein Expression System (Promega). The protein was purified using ProBond Purification System (Invitrogen), separated on a 7.5 % SDS-PAGE under non-reducing conditions, and then transferred to PVDF membrane. After blocking, the membranes were incubated with 10 mg/ml of *S. ricini* or *B. mori* HDLp in binding buffer (20 mM HEPES, 150 mM NaCl, and 2 mM CaCl2, pH 7.5) containing 0.5% BSA and then incubated with rabbit anti-*Sr*HDLp antibody. *Sr*LpR7-1 bound antibodies were detected with alkaline phosphatase conjugated goat anti-rabbit IgG (Invitrogen) and NBT/BCIP (Roche). The control reactions consisted of protein analyzed under reducing conditions and also with Lp antibody omitted.

General methods

Unless indicated otherwise, all molecular biology techniques were performed essentially as described (Sambrook *et al*. 1989). Protein estimation was performed with the BCA Protein Assay Kit (Invitrogen) using BSA as the standard. Tools from NCBI/ExPASy and SignalP 3.0 were used for nucleotide and amino acid analyses.

Results

Sequence analyses

The full-length cDNA of *Sr*LpR7-1 contained a 201 bp 5'-UTR followed by a 2598 bp ORF and a 1333 bp 3'-UTR (Accession number-KU936050). The putative start codon is preceded by in-frame stop codons, indicating that the sequence represents a full-length open reading frame. It encoded a protein of 865 aa with a predicted molecular mass of 94 kDa without a signal peptide and an isoelectric point (pI) of 4.24. The putative signal peptide is 22 aa residues with a predicted cleavage site between residues 22 and 23 followed by five domains, characteristics of the LDLR superfamily

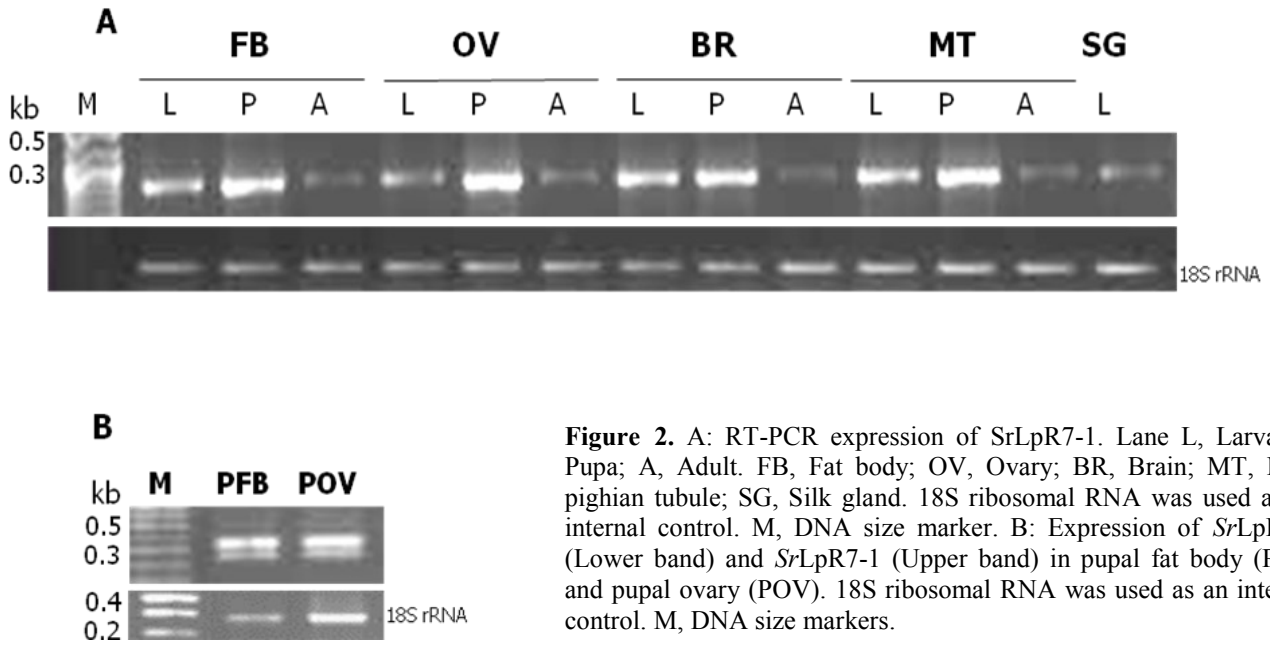

Figure 2. A: RT-PCR expression of SrLpR7-1. Lane L, Larva; P, Pupa; A, Adult. FB, Fat body; OV, Ovary; BR, Brain; MT, Malpighian tubule; SG, Silk gland. 18S ribosomal RNA was used as an internal control. M, DNA size marker. B: Expression of *Sr*LpR7-2 (Lower band) and *Sr*LpR7-1 (Upper band) in pupal fat body (PFB) and pupal ovary (POV). 18S ribosomal RNA was used as an internal control. M, DNA size markers.

(Figure 1A). Remarkably the first domain, the LBD consisted of seven cysteine-rich repeats in contrast to eight in most LpR and hence designated as *Sr*LpR7. The sequence analysis indicated that the first cysteine repeat is absent. The LBD was characterised by conserved SDE residues which are involved in disulphide bond formation and folding. The LBD was followed by an EGF-like domain with A, B, and C type cysteine -rich repeats containing five copies of F/YWXD sequence. The role of this domain is the acid dependent dissociation of ligand (s) in the endosomes. The third was the *O*-linked glycosylation domain with 49 amino acids containing *O*-linked sugar chains and phosphorylation sites, followed by a short transmembrane domain. The exact role of the *O*-linked sugar domain is not known but it is often characterized by deletions and insertions in LDLR family members. The transmembrane domain plays an important role in receptor recycling, as it passes between plasma membrane and various endocytic vesicles. The fifth cytoplasmic domain contained the conserved peptide motif FDNPVY required for receptor internalization via clathrin-coated pits. We have also obtained another splice variant, *Sr*LpR7-2, having a deletion of 27 amino acids in the *O*-linked sugar domain (Accession number- KU936051; Figure 1B). The two cDNAs encoded identical proteins except for an insertion/deletion of 27 amino acids. The *Sr*LpR7-1 showed a high degree of sequence similarity at the amino acid level with LpRs of other insects, such as *B. mori* (89.10 and 85.00 %; accession nos.- BAE71406 and XP_012551061, respectively), *G. mellonella* (87.40%, accession no.- ABF20542), *B. germanica* (68%, accession No.- CAL47126), *L. maderae* (67.70 %, accession No.- BAE00010) and *A. aegypti* (Ov- 66.53%, Fb- 61.50%; accession nos.- AAK72954 and AAQ16410, respectively).

Tissue distribution

Tissue and stage-specific mRNA expression levels were carried out by PCR using different primer pairs that amplify the two isoforms. As shown in Figure 2A, the *Sr*LpR7-1 transcripts were detected in all selected tissues at various developmental stages, with highest transcript levels seen in larval and pupal fat bodies, brain, Malpighian tubules and pupal ovary. Moderate levels of expression were observed in the silk gland and all adult tissues. The transcript level was low in non-vitellogenic ovary (Larval ovary) which was increased during the vitellogenic phase (Pupal ovary),

Table 1. Effect of RNAi on adult emergence, fecundity and hatching. The values shown are mean ± S.D. (n = 3).

Treatment	Day of Adult Emergence	Fecundity (No.)	Hatching %
dsLpR- injected	**18-19**	**210 ± 1.4**	**81± 2.3**
dsGFP-injected	**15-16**	**208± 2.0**	**80± 1.9**
Water-injected	**15-16**	**211± 1.9**	**79±2.3**
Normal	**15-16**	**208± 2.1**	**81±2.6**

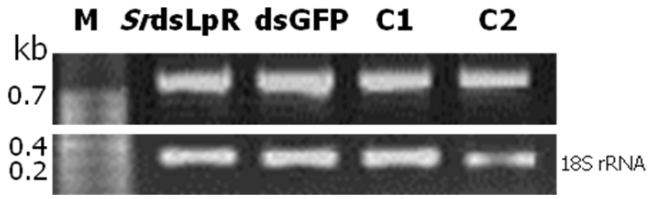

Figure 3. *Sr*LpR7 mRNA expression levels after RNAi. dsLpR, dsGFP, water injected (C1) and normal females (C2); 18S ribosomal RNA used as an internal control; M: DNA size markers.

coincident with the peak of yolk protein uptake. The mRNA expression of the *Sr*LpR7-2 showed low levels in most tissues (data not shown) except in pupal fat body and pupal ovary (Figure 2B).

RNAi

To determine the effects of RNAi of *Sr*LpR7, female pupae were injected with LpR dsRNA, GFP dsRNA and water as control groups. The results are presented in Table 1. We did not find considerable decrease in mRNA transcript levels in the experimental compared with the GFP and water-injected and normal females (Figure 3). However, the adult emergence was delayed for three days when compared to controls. Examination of ovaries revealed no abnormalities in any group which was followed by normal egg laying and hatching.

Ligand binding

The *Sr*LpR7-1 cDNA without its signal sequence was expressed and a protein of approximately 95 kDa was obtained. The protein was first incubated with Lps from *S. ricini* or *B. mori* and was subsequently detected using *S. ricini* anti-Lp antibody. The expressed receptor protein showed binding to Lp (Figure 4). Interestingly, the *Sr*LpR7-1 was also bound to *Bm*Lp and the *Sr*Lp antiserum reacted with *Bm*Lp (Figure 4). The cross reactivity of the *Sr*LpR to *Bm*Lp and the recognition of *Bm*Lp by *Sr*Lp antibody suggest the homology of structure at the amino acid level of these proteins between the two species. No binding was detected in the control reaction which was performed with SDS-PAGE under reducing conditions (Figure 4), indicating intact disulphide bonds are necessary for receptor binding. No binding was also detected in another control reaction in which Lp antibody was omitted (data not shown).

Discussion

We have cloned and characterized an LpR from eri silkworm that shares the typical modular domains, the hallmark of the members of LDLR superfamily. The

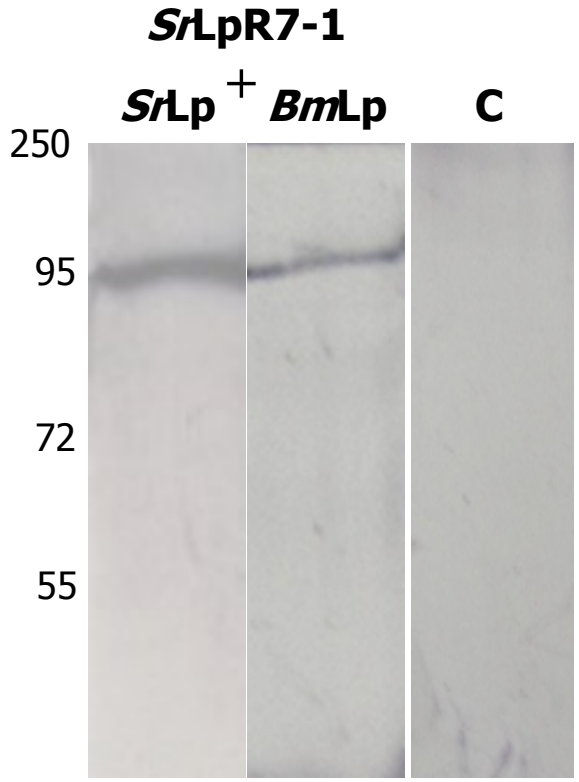

Figure 4. Functional expression of *Sr*LpR7-1 and its cross reactivity with *Bm*Lp. Lane 1: *Sr*LpR7-1 binds to *Sr*Lp; Lane 2: *Sr*LpR7-1 binds to *Bm*Lp; lane 3: Control showing no receptor binding. Numbers on the left indicate protein size markers in kDa.

LBD mediates the interaction between the receptor and the ligand, and usually consists of eight cysteine-rich repeats. While most LpRs contain eight cysteine-repeats in their LBDs and therefore identical to VLDLRs, the *Sr*LpR7-1 and 2 contain only seven such repeats and structurally resemble LDLRs. The LpRs of *A. aegypti* (fat body-specific) and *B. germanica* have also been reported to have seven cysteine-rich repeats (Cheon *et al*. 2001, Ciudad *et al*. 2007). A predicted *Bm*LpR isoform with seven cysteine repeats in LBD was found in the database (Accession Number: XP_012551061). As in *B. germanica*, the occurrence of seven cysteine repeats does not determine tissue or stage-specificity; *Sr*LpR7-1 was expressed in fat body, ovary and as well as other tissues and in all developmental stages. In *A. aegypti*, another splice variant of LpR specific to the ovary with eight cysteine repeats has been characterized (Seo *et al*. 2003). We could not isolate or amplify a full-length cDNA of *Sr*LpR with eight cysteine repeats from any tissues including ovary and fat body, whereas *Sr*LpR7-1 was frequently amplified indicating its abundance. The significance of the number of cysteine repeats in LBD is not known. In *D.*

melanogaster, lpr1 and *lpr2* are transcribed as multiple isoforms (having seven and eight cysteine repeats) and are essential for the cellular acquisition of neutral lipids. In addition, it is the presence of cysteine repeats in LBD and not the number that defines the ability of the receptor to mediate lipid uptake (Parra-Peralbo & Culi 2011). The variant form of *Sr*LpR is identical to the other except by the deletion of 27 amino acids in the OSD. This domain is the most divergent among five domains, with many deletions and insertions having occurred in the LDLR (Davis *et al*. 1986). The CD of *Sr*LpR contains an internalization signal, FDNPVY which is conserved in all vertebrate and invertebrate LDLRs. The alternative splicing and functional diversity are common features among the members of LDLR super family (Tveten *et al*. 2006). Compared to vertebrates, the functional specificities of LpR isoforms are not well understood. We have previously identified four splice variants of the *B. mori* LpR gene products, in which one, LpR4, was specific to the brain and possibly acts as a signalling receptor (Gopalapillai *et al*. 2006, 2014).

Transcripts of *Sr*LpR-1 were detected in all tissues tested by RT-PCR with the fat body, ovary, brain and Malpighian tubule being the highest sites of expression. The high level of transcripts in the pupal ovary suggests that the receptor is involved in the uptake of Lp into the ovary as vitellogenesis takes place in the pupal stage. Similar findings were reported from *B. mori* and *B. germanica* (Ciudad *et al*. 2007, Gopalapillai *et al*. 2006). In *A. aegypti*, the *Aa*LpROv is present during previtellogenic and vitellogenic stages whereas *Aa*LpRfb expression is restricted to the post-vitellogenic period (Cheon *et al*. 2001, Seo *et al*. 2003). Apart from most tissues, honey bee LpR mRNA has been specifically detected in the hypopharyngeal gland of adult females (Guidugli- Lazzarini *et al*. 2008). Expression of the LpR genes has been observed in a wide range of tissues including ovary, fat body, midgut, brain, Malpighian tubules, testis, embryo, muscle and silk glands (Tufail & Takeda 2009). Because of the functional diversity of Lps, the presence of LpR transcripts in a wide range of tissues is not surprising.

Lp plays an important role in lepidopteran vitellogenesis. Since *Sr*LpR7-1 was highly expressed in the vitellogenic ovary, we carried out RNAi experiments to determine its effect on ovarian development and reproductive success. In spite of having a delay in the transformation from pupa to adult moth, the dsLpR treated eri silkmoths showed normal fecundity and hatching. High or no silencing can occur at very different concentrations of dsRNA and lepidopteran insects often require relatively high dosage of RNAi molecules (Terenius *et al*. 2011). The concentration of injection was 50 µg dsRNA as lower concentrations were ineffective and higher concentrations (>50 µg, injected at a time) induced pupal mortality (data not shown). Hence, each dose of 50 µg dsRNA was administered at intervals. In a similar experiment, the same doses 50 µg dsRNA of vitellogenin receptor of *S. ricini* (*Sr*VgR) severally interfered with the ovarian development and resulted in poorly developed ovarioles (Manuscript under preparation). There is a mixed response to RNAi in lepidopteran insects and an accumulating body of literature has revealed that the efficiency of RNAi varies between different species and genes being targeted (Terenius *et al*. 2011). In *B. germanica* the treatment with 10 µg of ds*Bg*LpR reduced the lipophorin levels in the ovary but had no significant effect on ovarian development and fertility (Ciudad *et al*. 2007). The extension of pupal period by the dsLpR treatment in eri silkworm may indicate its involvement in the pupal-adult transformation. While no other reports are available on RNAi using LpR genes from other insects, further studies are required to establish the sensitivity to RNAi with LpR genes.

Unlike vertebrate counterparts, functional studies on LpRs are scarce. The recombinant receptor was successfully expressed and the results of the binding experiments indicated that *Sr*LpR7-1 bound to Lp of eri silkworm. Furthermore, the disulphide bonds in the receptor are required for binding, as no binding was observed under reducing conditions. Our results are consistent with the bindings of mosquito *Aa*LpRfb and *Aa*LpRov (seven and eight cysteine repeats respectively); both receptors bind to mosquito Lp, despite the differences in their LBDs (Cheon *et al*. 2001, Seo *et al*. 2003). We further demonstrate that *Sr*LpR not only binds to *Sr*Lp but also to *Bm*Lp and the polyclonal antibodies of *Sr*Lp react with *Bm*Lp. The cross-reactivity of *Sr*Lp/LpR indicates they are biochemically and immunologically similar to *Bm*Lp/LpR, a closely related species. The amino acid sequence identity between the two LpRs is 89.10 %. There is only one report available on the cross-reactivity of LpR in which *Manduca sexta* LpR did not bind to human low-density lipoprotein, in spite of its structural similarities with vertebrate lipoprotein receptors (Tsuchida & Wells 1990). However, cross-reactivity of LDL receptors among vertebrates has been reported (Beisiegel *et al*. 1981).

Conclusions

The present study provides the first evidence of the presence of an LDLR homolog in a lepidopteran insect. We have further shown that the *Sr*LpR7 is able to cross-interact with Lp of another lepidopteron species.

Future studies on the protein expressions of LpR variants and their ligand specificities will add to a better understanding of the functionality of this ancient receptor.

Competing interests

The authors state no conflict of interest.

Authors' contributions

R. Bala, U. Saba, M. Varma, D. S. Thomas, D. K. Sinha were involved with experiments, G. Rao, K. Trivedy and V. Kunjupillai were assisted in data analysis, R. Gopalapillai was involved in project planning, evaluation of the data and writing of the paper.

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