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A tapeworm molecule manipulates vitellogenin expression in the beetle *Tenebrio molitor*

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Abstract

Metacestodes of *Hymenolepis diminuta* secrete a molecule that decreases vitellogenin (Vg) synthesis in the beetle host, *Tenebrio molitor*. The 5608 bp *T. molitor* Vg cDNA represents a single-copy gene encoding a single open reading frame of 1821 amino acids with a predicted molecular mass of 206 kDa. Northern blot analysis revealed detectable levels of transcripts only in adult females. *In vivo*, Vg mRNA abundance was significantly higher in fat bodies from infected females compared with control females at all but the earliest time point. *In vitro*, Vg mRNA abundance was significantly increased in fat bodies incubated with live stage I–II parasites. The apparent conflict between increased Vg mRNA abundance and decreased Vg protein in fat bodies from infected females is discussed.

Keywords

Tenebrio molitor; *Hymenolepis diminuta*; vitellogenin; mRNA abundance

Introduction

A decrease in reproductive output is commonly found in insect intermediate hosts that are parasitized (Hurd, 2001). We are using the relationship between the rat tapeworm, *Hymenolepis diminuta* and its intermediate beetle host, *Tenebrio molitor*, to explore a parasite strategy to manipulate intermediate-host resource management in a way that decreases the resources devoted to host reproduction. Changes in both fecundity and fertility have long been observed in female *T. molitor* parasitized by *H. diminuta*. Reductions in both egg production and egg viability are associated with two major events. First, there is a decrease in synthesis of vitellogenin (Vg) by the fat body and secondly, the uptake of circulating Vg by the ovaries is diminished (Webb & Hurd, 1996). Developing parasites produce a manipulator molecule that directly affects insect vitellogenesis by decreasing Vg synthesis in the fat body of female insects both *in vivo* and *in vitro* (Webb & Hurd, 1996, 1999). The parasite molecule is stage-specific in that significant decreases in Vg synthesis are typically associated only with early (stage I–II) metacestodes (Webb & Hurd, 1999). We have shown that this molecule is secreted by incubating fat body tissue from female beetles in medium conditioned by parasites (Hurd, unpublished). Briefly, fat body incubation medium was incubated with early (stage I–II) metacestodes (100 µl per 100 parasites) at 26 °C for 4 h. When fat body tissue was incubated in this conditioned medium, Vg production was reduced by 61% compared with fat body tissue incubated in control medium, reflecting

a significant difference ($P = 0.04$). Although we have been unable to purify this manipulator molecule to date, reverse phase high-performance liquid chromatography of an acid-extract of stage II metacystodes has revealed the presence of a biologically active peak containing two compounds. Our data suggest that one of these compounds is a short peptide of about 200–250 Da. Despite extensive investigations, we have been unable to sequence this peptide, which appears to be N-terminally blocked (Hurd, unpublished).

We investigated previously the possible role of apoptosis (programmed cell death) in the beetle fat body as a mechanism by which Vg synthesis might be reduced (Warr et al., 2004). Our data provided evidence that *H. diminuta* infection causes apoptosis in a proportion of the fat body cells. While this may contribute to the reduction in Vg synthesis *in vivo*, our *in vitro* studies showed that apoptosis was not rapidly induced by the presence of live parasites. We therefore concluded that apoptosis was not directly induced by the manipulator molecule that down-regulates Vg synthesis. Consequently, we investigated whether the decrease was associated with transcriptional regulation through reduced abundance of Vg mRNA. In mosquitoes, infection with malaria parasites causes a significant decrease in Vg mRNA abundance, changes in the titre of Vg circulating in the haemolymph and a decrease in Vg content in the ovaries (Ahmed et al., 2001). The hypothesis to be tested in the present study was that *H. diminuta* infection in *T. molitor* would similarly affect Vg mRNA abundance. As there was no information available on Vg gene(s) in *T. molitor*, we first cloned and characterized the complete Vg cDNA so that our hypothesis could be tested.

Over the past decade, there has been a steady increase in the number of insect Vg genes that have been cloned and sequenced (Chen et al., 1994; Romans et al., 1995; Lee et al., 2000; Tufail & Takeda, 2002). Analysis of their deduced amino acid sequences has identified a number of common characteristics allowing them to be aligned confidently along their entire length. These include a highly conserved GL/ICG motif and a series of cysteine residues at conserved locations towards the C-terminus of the protein (Romans et al., 1995; Lee et al., 2000). A similar motif (CGLCG) is also common in the C-terminus of Vg proteins in a number of vertebrate and nematode species and can be aligned with the GL/ICG motif of insects (Hagedorn et al., 1998).

Although some of the molecular events involved in Vg gene activation have been studied in insects, most work has focused on hormonal control by 20-hydroxyecdysone and little extends beyond this to investigate the specific mechanisms whereby Vg genes are regulated. One exception is the Vg gene from the mosquito, *Aedes aegypti*, where transcriptional regulation has been studied in detail, revealing three distinct promoter domains. A proximal region, responsible for tissue and stage specificity, carries binding sites for EcR/USP, GATA, C/EBP and HNF3/fkh. Upstream of this, a median domain, carrying binding sites for ecdysone response factors E74 and E75, is required for hormonal enhancement. Further upstream, a distal domain containing binding sites for multiple GATA factors is required for the high levels of Vg expression seen *in vivo* (Kokoza et al., 2001). More recently, the *A. aegypti* Vg gene promoter has also been shown to carry multiple binding sites for iso-forms of the *Broad* gene that encode C2H2-type zinc-finger DNA binding proteins, acting as transcriptional regulators of the 20-hydroxyecdysone cascade (Chen et al., 2004). In stark contrast, nothing is known of the regulation of the *T. molitor* Vg gene.

We have previously shown that Vg synthesis in *T. molitor* is decreased both *in vivo* and *in vitro* by a molecule secreted by *H. diminuta* (Webb & Hurd, 1996, 1999; Hurd, unpublished). Here, we study Vg mRNA abundance to determine whether the parasite manipulator molecule can act to alter the expression of the Vg gene. This was accomplished by comparing Vg mRNA abundance profiles between infected and non-infected beetles at various time-points post-emergence.

Results

Characterization of the Vg precursor cDNA from *Tenebrio molitor*

The 3' end of the Vg gene from *T. molitor* was isolated by 3'-RACE from a cDNA template (Lee et al., 2000; Piulachs et al., 2003). Reactions were carried out with both GLCG and GICG degenerate forward primers but only the former produced a band of the expected size (600–700 bp; results not shown). This was re-amplified in a nested reaction to improve yield and specificity and the product cloned and sequenced. The resulting 637 bp sequence corresponded to the 3' end of the *T. molitor* Vg precursor gene. To obtain the remaining 5' portion of the cDNA we used 5'-RACE and the complete 5608 bp Vg cDNA sequence was deposited in the DDBJ/EMBL/GenBank databases (accession no. AY714212). The cDNA included a 5'-UTR of 30 bp, 3'-UTR of 98 bp, two putative polyadenylation signals (AATAAA) located either 74 or 78 bp downstream of the stop codon (TGA, nucleotides 5494–5496) and a poly(A) tract either 12 or 16 nucleotides downstream of the polyadenylation signal.

Conceptual translation of the cDNA identified a single open reading frame of 1821 amino acids with the first 16 residues corresponding to a signal peptide (Fig. 1). The translated sequence was aligned (Clustal W) with all 31 complete insect Vgs available to date to reveal highly conserved residues and motifs (Fig. 1). Phylogenetic analysis (data not shown) supported previous reports that Vg relationships reflect the accepted classification of insects (Piulachs et al., 2003). Notable features of the *T. molitor* Vg include the anticipated GL/ICG motif (residues 1648–1651) and 32 residues that are identical across all 31 insect Vgs (Fig. 1), including four cysteines at the C-terminus that have been described previously (Romans et al., 1995; Hagedorn et al., 1998; Lee et al., 2000). The closest match (identity 34%, similarity 54%) was with the Vg protein of the boll weevil, *Anthonomus grandis* (Trewitt et al., 1992), the only other complete Coleopteran sequence available. In a comparison of insect, vertebrate and nematode Vgs, Chen et al. (1997) described five subdomains that could be aligned with confidence. For *T. molitor* Vg, these subdomains I–V correspond to residues 22–339, 427–846, 883–1072, 1480–1687 and 1743–1821, respectively. We note that they reflect the majority of conserved regions in our analysis of insect Vgs but that they omit three residues conserved among all 31 sequences, including an identical serine [S₍₁₂₅₃₎] and cysteine [C₍₁₃₅₉₎].

Tufail et al. (2000) identified a conserved DGXR motif, which was described as being located 18 residues upstream of GL/ICG in all insects except the cockroach, *Lucophaea maderae*, where it was reportedly absent. Using all 31 complete insect Vgs and a partial Vg sequence (U63328) from the spruce terminal weevil, *Pissodes strobi* (Leal et al., 1997) we noted instead that, with one exception, this motif is located 17 residues upstream of GL/ICG in the Lepidoptera, 18 residues upstream of GL/ICG in the Hymenoptera, Orthoptera, Hemiptera and Diptera and 19 residues upstream of GL/ICG in the Coleoptera (Fig. 2). The one exception is the Hymenopteran *Solenopsis invicta* Vg2, where the motifs are separated by 19 residues. The final residue of the motif is [R] in all sequences except *S. invicta* Vg1 and Vg3 [H] and *T. molitor* [T] and a divergent motif is clearly recognizable in *L. maderae* where [EN] replaces [DG]. The reasons for this variation in sequence and location are unclear. The C-terminal alignment also revealed a series of identical residues, including an aspartic acid [D] nine residues downstream of GL/ICG, four cysteines and a proline (Fig. 2). We note that the GL/ICG motif itself is not invariant, with a divergent GLAG motif evident in *Riptortus clavatus* (Fig. 2).

Genomic organization of the *Tenebrio molitor* Vg gene

The copy number of Vg genes varies from one to three among the 23 species for which data exist and duplication is an apparently common event (Scott et al., 2005). Southern blot analysis of pooled genomic DNA from *T. molitor* larvae indicated that the Vg gene was present as a single copy per haploid genome. A 666 bp fragment corresponding to nucleotides 2129–2794 of the Vg cDNA hybridized at high stringency to single fragments of approximately 3.6 kb, 2.4 kb, 5.0 kb and 1.9 kb in genomic DNA digested with *XmnI*, *BamHI*, *SalI* and *HindIII*, respectively (Fig. 3). Phosphorimages of the membrane taken after a series of lower stringency washes failed to detect any other hybridizing fragments that might have indicated additional Vg genes in the *T. molitor* genome (data not shown). In a separate Southern blot analysis, a 637 bp fragment corresponding to nucleotides 4972–5608 at the 3′ end of the Vg cDNA also hybridized to single fragments of 2.6 kb and 3.5 kb in genomic DNA digested with *BamHI* and *XmnI*, respectively (data not shown), consistent with the interpretation of a single-copy Vg gene in *T. molitor*.

Transcriptional profile of the *Tenebrio molitor* vitellogenin gene

Northern blot analysis of *T. molitor* mRNA abundance revealed no detectable expression of Vg transcripts in either larvae, male or female pupae, or male adults (Fig. 4A). The profile of Vg mRNA abundance indicated that Vg gene expression was continuous throughout the development of the female adult beetle, up to day 15 post-emergence after which no further measurements were taken (Fig. 4A,B). Following normalization of Vg mRNA abundance to an internal 18S rRNA control, ANOVA revealed no significant differences in Vg mRNA abundance across the female adult profile ($F_{6,19} = 1.88$; $P = 0.137$).

Impact of parasite infection of Vg mRNA abundance in vivo

The abundance of Vg mRNA was compared in both control and infected females at various time points post-emergence. As the effect of the parasite manipulator molecule is seen early in infection (Webb & Hurd, 1996, 1999), no mRNA samples were analyzed beyond 15 days post-emergence. Northern blot analysis revealed that Vg mRNA abundance was higher in the fat body tissue from infected beetles at all time points post-emergence, compared with the fat body tissue of the control beetles (Fig. 5A). These differences were significant ($F_{9,30} = 2.74$; $P < 0.05$) for all time points except day 4 post-emergence.

Impact of live parasites on Vg mRNA abundance in vitro

The abundance of Vg mRNA in fat body tissue that had been incubated in the presence or absence of live parasites was investigated (Fig. 5B). In comparison with control fat body incubations or fat bodies incubated with mature parasites (developmental stage V–VI), there was a significantly higher ($F_{2,15} = 28.75$; $P < 0.001$) abundance of Vg mRNA transcripts in fat body tissue incubated with live stage I–II parasites (Fig. 5B). We conclude from this that the manipulatory molecule is secreted by live, early stage parasites.

Discussion

We consider here the manipulation of Vg gene expression in *T. molitor* by a secreted molecule from the tapeworm *H. diminuta*. Initially, a 637 bp fragment of the *T. molitor* Vg gene was cloned by 3′-RACE using gene-specific degenerate primers targeted at the GL/ICG motif conserved within the C-terminus of all insect Vg precursors (Lee et al., 2000; Piulachs et al., 2003). Such primers are very short (11 bp) but appear to work effectively in this context because of the high abundance of Vg transcripts in the fat body tissue of insects. cDNA sequencing was completed by 5′-RACE using reverse primers designed to the 3′ sequence. Vg genes appear to be commonly duplicated within insect genomes (Scott et al., 2005) though this is not universal. Our data indicate that the *T. molitor* Vg gene is present as

a single copy per haploid genome. Following conceptual translation of the *T. molitor* Vg cDNA, BLASTP analysis confidently aligned the protein sequence with other insect Vgs through sequence conservation at numerous sub-domains. The closest match was with the Vg protein of the boll weevil, *A. grandis* (Trewitt et al., 1992), which is consistent with the phylogenetic analysis of Piulachs et al. (2003).

Insect Vgs are generally encoded by mRNAs of 5.5–6.5 kb and translated as polypeptides of about 200 kDa (Wyatt & Davey, 1996). The 5608 bp mRNA of the *T. molitor* Vg encodes a polypeptide with a predicted molecular mass of 206 kDa. Most insect Vg precursors are cleaved once (within the fat body) by subtilisin-like proprotein endoproteases to produce two subunits. Cleavage immediately follows an R/KXXR motif, which may in some cases be flanked by polyserine tracts (Barr, 1991; Sappington & Raikhel, 1998) but which may not be followed immediately by L, I or V (Denault & Leduc, 1996). The *T. molitor* Vg has 10 putative cleavage sites (Fig. 1), although only two of these would be supported by published observations of cleavage products. Both Harnish & White (1982) and Webb & Hurd (1995) detected large subunits of 154 and 145 kDa and small subunits of 56 and 45 kDa, while Michalik et al. (1996) detected a single large subunit of 158 kDa and two small subunits of 56 and 45 kDa. We note that both *T. molitor* and *A. grandis* have recognizable R/KXXR motifs in almost identical locations (*A. grandis*, RRFR_[359–362] or RFRR_[360–363]; *T. molitor*, KSSR_[365–368] or RTKR_[368–371]). These motifs are not flanked by obvious polyserine tracts, although there is some evidence of serine richness in the residues immediately preceding each site. Cleavage of the *A. grandis* Vg at this site has been determined experimentally (Heilmann et al., 1993) yielding subunits of about 47 and 160 kDa, which might be compared with *in silico* predictions for molecular weight (ExPASy) following cleavage at this site of about 40 and 165 kDa. Cleavage of the *T. molitor* Vg at KSSR_[365–368] or RTKR_[368–371] would also yield predicted subunits of about 40 and 165 kDa. It is possible that the variant large and small subunits might result from post-translational modifications, such as glycosylation, phosphorylation or sulphation, all of which have been observed in *A. aegypti* Vg, or as artefacts of cleavage by proteases during sample preparation (Sappington & Raikhel, 1998).

Alternatively, cleavage of the *T. molitor* Vg at the C-terminal motif KCKR_[1344–1347] would yield fragments of 54 and 152 kDa, which are also consistent with the experimentally determined subunits. In this case, further cleavage of the small subunit at one of the four distal C-terminal motifs (Fig. 1) could generate the 45 kDa peptide and further cleavage of the large subunit at KKMR_[124–127] (Fig. 1) could generate the 145 kDa peptide. Cleavage at a C-terminal R/KXXR motif is unusual among insects, having been reported only for the gypsy moth, *Lymantria dispar* (Hiremath & Lehtoma, 1997). Interestingly, *Lymantria* Vg is cleaved at KAKR_[1459–1462] and the authors highlight the absence of flanking serine-rich regions, a property shared by the KCKR_[1344–1347] motif in *T. molitor*.

Expression of the *T. molitor* Vg gene is restricted to adult females with no evidence of significant variation in Vg mRNA abundance across females of different ages. This correlates with egg production in this species, which is continuous and characterized by asynchronous development of oocytes in adjacent ovarioles (Ullmann, 1973; Gerber, 1975). It was previously reported that *in vitro* incubation of fat body tissue from male *T. molitor* yielded a small amount of Vg, approximating to 10% of that produced by fat body tissue from females (Webb & Hurd, 1996). Although rare, a number of other male insects have been reported to produce Vg in the absence of exogenous hormonal stimulation (Webb & Hurd, 1996). No Vg mRNA was detected by Northern blot analysis in male fat bodies in this study, despite the use of sensitive phosphorimaging, although low-abundance mRNA might be detected by more sensitive technologies such as real-time quantitative PCR.

Contrary to our expectations, Vg mRNA abundance in the fat body of infected females was significantly higher than that from uninfected females at all but the earliest time point tested. An *in vitro* assay had previously been established to test the direct action of the secreted parasite molecule of fat body Vg synthesis (Webb & Hurd, 1999). An incubation time of 4 h was established as sufficient to demonstrate significant reductions in Vg synthesis, either by the addition of live parasites or a parasite extract (Webb & Hurd, 1996, 1999). In the present study, incubation with early (stage I–II) parasites resulted in a significant increase in Vg mRNA abundance. Thus, in contrast to malaria-infected mosquitoes, *H. diminuta* does not cause a reduction in Vg mRNA abundance in *T. molitor*. Clearly, the observed decrease in Vg protein in infected females is not the result of a decrease in Vg mRNA abundance. These data present an apparent contradiction. The manipulator molecule from early stage parasites causes both a significant increase in Vg mRNA abundance in the fat body and a significant decrease in the levels of Vg protein both *in vivo* and *in vitro*. One possible interpretation is that the parasite molecule alters the storage of Vg mRNA, with more transcripts being stored than translated. In locusts, adipokinetic hormone (AKH) is involved in the negative control of Vg synthesis and is known to act at the protein level (Moshitzky & Applebaum, 1990; Glinka et al., 1994). There has been a suggestion that AKH is involved in the shift between mRNA fates, with transcripts either being sequestered into storage particles or translated into protein (Wyatt et al., 1995). So, in terms of a possible post-transcriptional mode of action, it is conceivable that the *H. diminuta* molecule could be mimicking an endogenous insect hormone or neuropeptide such as AKH, which normally regulates the production of Vg (Webb & Hurd, 1999). In this context, it has been shown that certain parasites, for example *Spirometra mansonoides*, can synthesize and release host-like molecules (Halton et al., 1994). More relevant to this study is the detection of peptides that exhibit AKH-like bioactivity in the nematodes *Panagrellus redivivus*, *Ascaridia galli* and *Ascaris suum* (Davenport et al., 1991, 1994). We speculate that the increase in Vg mRNA abundance coupled to a decrease in Vg protein synthesis might result from a positive feedback mechanism that recognizes falling Vg synthesis and attempts to compensate by increasing Vg gene transcription.

Reduction in the reproductive output of insect hosts is a life history trait that is commonly associated with parasitic infections. There has been much debate in the literature about whether such changes are simply by-products of infection or adaptive manipulation by the host or parasite (Hurd, 1998; Poulin & Combes, 1999). In our host–parasite association, *H. diminuta* is able directly to manipulate the synthesis of Vg in the fat bodies of female *T. molitor*. It is of particular interest that the parasite specifically reduces the production of Vg but not of other proteins in the fat body (Webb & Hurd, 1996). The survival of female insects does not rely on the production of eggs, which places a heavy demand on the resource allocation of amino acids. Thus, there may be an advantage to reducing egg production such that nutrients may be redistributed and better used elsewhere. An infected female beetle producing a normal quantity of eggs may not be viable, so a shift in resource allocation away from egg production may provide a better environment for both host and parasite (Webb & Hurd, 1996). In addition to these reductions in egg production, it has been demonstrated that infection with *H. diminuta* extends the life-span of female *T. molitor* by about 50%. This increase in host longevity may provide a selective advantage to the parasite by providing more opportunity for the host to be predated and therefore parasite transmission to occur (Hurd, 2001; Hurd et al., 2001).

Experimental procedures

Maintenance of parasite and host

Eggs from adult *H. diminuta* (ARME strain) were obtained from faecal pellets of male Wistar rats by salt flotation (Hundley & Berntzen, 1969) and stored at 4 °C in distilled water

for a maximum of 2 weeks. *T. molitor* (Rice University strain) were kept in plastic tanks at 26 °C, 60–70% relative humidity on a 12 : 12 h light/dark cycle and fed wheat bran *ad libitum* with a weekly potato supplement to provide moisture. Pupae from the stock colony were sexed (Bhattacharya et al., 1970) and maintained at 26–27 °C, 60–70% relative humidity until emergence. Adults were placed in Petri dishes at a ratio of 10 females: three males and starved for 48 h. On day 3 post-emergence, control populations were fed apple pulp alone and infected populations were fed apple pulp mixed with parasite eggs, such that the age of the beetle is 3 days more than that of the parasite. Apple pulp was removed after 24 h and all insects fed subsequently on wheat bran (*ad libitum*) supplemented with apple slices every third day.

Characterization of the *Vg* precursor cDNA from *T. molitor* mRNA was purified from total RNA (TRIZOL reagent, Invitrogen, Paisley, UK; Nucleotrap, Takava Bio Inc, France) and subjected to 3'-RACE (GeneRacer; Invitrogen). Briefly, mRNA was reverse-transcribed (SuperScript II) with an oligo dT primer to create first strand cDNA with known primer binding sites at the 3' end. This was amplified using gene-specific, degenerate forward primers and a generic reverse primer. The forward primers (Lee et al., 2000) were based on GLCG (5'-GGICTSTGYGG-3') or GICG (5'-GGIATYTGYYG-3') and the reverse primer was the GeneRacer 3' primer (5'-GCTGTCAACGATACGCTACGTAACG-3'). PCR reactions [50 µl; 0.2 µM primers; 0.2 mM dNTPs; 2 mM MgSO₄; 2.5 U high fidelity Platinum *Taq* DNA polymerase (Invitrogen)] were cycled [94 °C, 2 min; (94 °C, 30 s; 55 °C, 1 min; 68 °C, 2 min) × 30; 68 °C, 10 min] in a PTC-200 cyclotherm (MJ Research, Bio-Rad Laboratoire, MA, USA). Reaction products were reamplified with a gene-specific nested reverse primer to increase sensitivity and specificity. PCR products were excised from 1% agarose TAE gels, purified (GeneClean, Q-BIOgene, MP Biomedicals, CA, USA) and cloned into pCR4-TOPO (Invitrogen) for sequencing. 5'-RACE used reverse primers designed to the 3' sequence. mRNA was reverse-transcribed with Vit5race (5'-GCAACGGACGAAGAGTGAAGCAAACCT-3') and amplified with the gene-specific Vit5Nrace (5'-CAGCAGCGCTCTCGTCTGTGAAC-3') and the generic GeneRacer 5' primer (5'-CGACTGGAGCACGAGGACACTGA-3') using the conditions described above but following the manufacturer's cycling parameters with an annealing temperature of 65 °C and extension time of 7 min. The purified product (≈ 5 kb) was cloned into pCR 2.1 TOPO (Invitrogen) after a 20 µl overnight ligation using undiluted salt solution and subsequent cleanup with Pellet Paint co-precipitant (Novagen Merck Biosciences, Darmstadt, Germany). The insert was sequenced on both strands using M13 primers followed by the internal primers Vitfwd1 (5'-GTCAACACTAACTCACCTAACA-3'), Vitfwd2 (5'-ATAGTAATGCAGAAGCCTCAC-3'), Vitrev1 (5'-ATCTCAGCTCGACCATACTC-3') and Vitrev2 (5'-GAGCGGATCTTGACTACGAA-3').

Southern blot analysis of the *Tenebrio molitor* genome

Genomic DNA was isolated using a salting out procedure (Miller et al., 1988). Briefly, four larvae were crushed in liquid nitrogen and resuspended in lysis buffer (500 mM Tris-HCl, pH 9.0; 20 mM EDTA; 10 mM NaCl) containing 0.5 mg/ml proteinase-K and 1% SDS. Preparations were incubated at 48 °C for 2 h and polypeptides were salted out of solution using saturated NaCl. Genomic DNA was ethanol precipitated, washed repeatedly with 75% ethanol and resuspended in 300 µl water. Following digestion with either *Xmn*I, *Bam*HI, *Sa*I or *Hind*III, approximately 10 µg of genomic DNA was separated on 1% agarose in TAE buffer and blotted on to Hybond-N+ (Amersham Biosciences GE Healthcare, Bockinghamshire, UK). Membranes were probed with a 666 bp fragment corresponding to nucleotides 2129–2794 of the *Vg* cDNA, radiolabelled to high specific activity with ³²P by random priming. Membranes were washed at increasing stringency from [5 × SSC; 0.1% SDS; room temperature] to a final stringency of [1 × SSC; 0.1% SDS; 68 °C] in order to

detect weakly hybridizing homologous sequences. All membranes were exposed to phosphor screens (Cyclone, Perkin Elmer) for between 2 and 48 h and the resulting images interpreted using Optiquant software (Perkin Elmer, MA, USA).

Northern blot analysis of *Tenebrio molitor* Vg mRNA abundance

Fat body tissue from individual infected and non-infected female beetles was dissected at the required age (days 1, 3, 4, 6, 9, 12 or 15 post-emergence) and total RNA isolated with TRIZOL reagent (Invitrogen). To measure the effect of live parasites *in vitro*, fat body tissue from individual uninfected females at 6 days post-emergence was dissected and incubated for 4 h at 26 °C with 10 live parasites (early stage I–II or late-stage V–VI) or with incubation medium alone, prior to isolation of total RNA. Total RNA was fractionated on denaturing 1% agarose-formaldehyde gels and transferred to Hybond-N+ membranes (Amersham Biosciences). Membranes were probed with a 637 bp fragment corresponding to nucleotides 4972–5608 at the 3' end of the Vg cDNA, radiolabelled to high specific activity with ³²P by random priming. Membranes were washed at increasing stringency and exposed to phosphor screens (Cyclone, Perkin Elmer) for between 2 and 48 h, with the resulting images interpreted using Optiquant software (Perkin Elmer). For normalization, membranes were stripped by washing in 0.1% SDS at 80 °C for 20 min and re-probed with a 200 bp 18S rRNA control fragment generated by RT–PCR using generic eukaryotic 18S primers (Applied Biosystems CA, USA). Final washing stringencies for the Vg and 18S probes were (2 × SSC; 0.1% SDS; 42 °C) and (0.1 × SSC; 0.1% SDS; 47 °C), respectively.

Statistical analysis

Data sets were tested for normality (Anderson–Darling) prior to one-way ANOVA (Minitab). Where significant differences ($P < 0.05$) were observed, individual means were subjected to unplanned comparisons (Tukey's method; family error rate = 0.05).

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MWSPILLCLI	VAFAYASEHP	AWEENKLYTY	EVRGRSFASL	NQISNVNSGI	LIKARLAVQL	60
NEDGKLGGLVI	TDAQYVQINR	ELSNWETDI	PDSEVNYEPL	DGLSYTPFEI	ELNKGAIKDI	120
FVE KKMR NWE	VNMVKGIVSQ	LQLDTKASNL	MVSSINILTQ	DDSNTAVFKT	TEETVTGVAE	180
TLYEIHYPYN	YVLQTPWV	PQKELIHNDQ	VIEVVKHKNY	TYSRQVLPS	YHADFEIGHG	240
YQPQNKIADF	MSRTSVSQAV	VTGSLKAFVI	QSSVTTEEIV	LRPTLADQQK	GSVNTKLTIT	300
LSSVLAPLDQ	DKLSLSDPVG	VG VYGYNNP	FGTDNGRPA	DPYDHSVPDV	SSESQSADSE	360
~NSYE KSSPTK R	STKYRKDVT	KKLNFAASYQA	DNLDSEKWHQ	KKPKINKAPA	SPLLPTVAH	420
GGQAIKKQQN	IVETVRKLAD	QVGGEFQDPN	EILRQHTVGK	FVTLVSLVRT	MDHEEVNKVA	480
TQLYPASLGQ	SSPAWVAYRD	AVAQSGTSPA	LLNIKEWIKS	GKVS GREAAE	VIATANAPR	540
APTEQHIKEF	FEIKDEDIL	SKPYVNESAI	LSYTDLVYKV	HSNAEASQ GK	FPCTVSDPSI	600
LKVGQDFVRK	TVIPHLTRKL	HEAISKADTR	KIHVYIRALG	NVGQYILEA	FEPYLEGTTK	660
ASHFORVLMV	TALDRLVESN	PTVGRSVFYK	IYRNP SEAE	VRVAAVFQLM	RTNPPADMLQ	720
RMASYTNVDS	SNYVNAAVKS	SIESASQLEE	ARLAPLREAA	ESAKPLLTTK	EFVGVQSQGY	780
~*~*~*~*~*~*	PGYEQYLQSF	GGEDYAVPKG	IKYSLSKVFG	GVSQPVLMQ	AMTSSVEDLI	840
NVYQQQTKEY	QDKLREKQNA	EVGSRNPWIS	ENTAKRLDIK	NEQREQLGN	LYLQANAWQE	900
IFSFNDHTLA	NLSQVVQELE	EEFSKGKSYK	YNKMLIGQES	SLALPTALGL	PFVYVYDRPA	960
LVRIQGDVKA	HAEPRISTEG	HIQKPDRVHA	EFEIKATVTG	RVQTQLAIIT	PFNHQRSSAG	1020
YIKNYQINVP	LSGKLELVNQ	KNTVKVEVQN	PAVNQEKIRL	AHYSSWPYTS	QGDVFSRPE	1080
LNIIRSKNPY	MINQIFGQKS	TGVALYLHIT	SDEIIDTAFI	YDKLRSQDPL	SAILSPWVDD	1140
NIQYVHVDVG	IDGSLSTAQQ	VNAEFIYRKE	YQPEKPSTNN	QEDDQEPDPK	PDEAEELDNI	1200
PPYPDDPERR	INAFDLKLRG	QIRSSSLYAA	DATAEFLGPS	KMKYSATVGI	SKSNVSPRGR	1260
FMALFRQLEK	QNPKTLAYLH	GWSYIINTNG	LNMDYAMKMN	PVASFRVKGL	LDREERLSKA	1320
SSDIDFFKSK	ERVHYLKQLE	DYK KCKRE MQ	EGNNQLPVCA	KMTALANLLD	KFTVRTKYS	1380
MDPKVINATY	TAYSVLRHSL	YPRIHENIVN	RDFDGHLDVD	GQFSPDLHAL	NVSVRSEYGR	1440
AEIQNVPMGP	WYRQLLVQQP	VFHAKTRLAA	QAYKYDTYRP	ICVIDKLSAS	TFDNKTYPAR	1500
LRPEWTVALQ	YIPRPSNEK	GKPYQTVLEQ	LTQQIESYV	YVRAPQNSNS	QKEVLIALQM	1560
PSTQGKPATI	SMKPRR SANQ	PPRVYVNDQE	VIPKLDISAD	FYNGNIQIYR	LPNNELKVEV	1620
HDAFYVIF DG QT	VKLTAVNS	KFRDAS GLC GT	TFDGEQETD	FLAPNNCILH	KPVNFVQSYT	1680
~*~*~*~*~*~*	~*~*~*~*~*~*	~*~*~*~*~*~*	~*~*~*~*~*~*	~*~*~*~*~*~*	~*~*~*~*~*~*	1740
LFWLNRQYTT	RRAAAQQQEE	EQKCYQKKVT	FANYISNRDA	GRTDR THE RQ SR	SDSCSRMQT	1740
QYMEQGNEVC	FTLRPLPLCK	DGCAPRATVS	KQVDVYCASR	SMRS VGVMN	EILKGASPDF	1800
~*~*~*~*~*~*	~*~*~*~*~*~*	~*~*~*~*~*~*	~*~*~*~*~*~*	~*~*~*~*~*~*	~*~*~*~*~*~*	1821
SEQTAS RKVR	MEVPQSCMPR	H				

Figure 1.

Conceptual translation of the *Tenebrio molitor* Vg cDNA showing predicted signal sequence (bold), putative R/KXXR cleavage motifs (open boxes) and conserved DGXR and GL/ICG motifs (reverse highlight). See text for details. Highly conserved residues (100% identity = *, > 90% identity = -; high similarity as defined by Clustal W = :) across all 31 available insect Vgs are indicated below each line. Sequences included were: *Aedes aegypti* VgA1, Q16927; *Aedes aegypti* VgB, AAQ92367; *Aedes aegypti* VgC, AAQ92366; *Anopheles gambiae* G3 Vg1, AAF82131; *Anopheles gambiae* PEST Vg1, XM_313104.2; *Antheraea pernyi*, BAB16412; *Antheraea yamamai*, BAB32640; *Anthonomus grandis*, 283605; *Apis mellifera*, CAD56944; *Athalia rosae*, 7522163; *Blattella germanica*, CAA06379; *Bombyx mandarina*, BAB32642; *Bombyx mori*, Q27309; *Encarsia formosa*, AAT48601; *Graptopsaltria nigrofusca*, BAA85987; *Leucophaea maderae*, BAD72597; *Lymantria dispar*, 11277187; *Periplaneta americana* Vg1, Q9U8M0; *Periplaneta americana* Vg2,

Q9BPS0; *Pimpla nipponica*, AAC32024; *Plautia stali* Vg1, BAA88075; *Plautia stali* Vg2, BAA88076; *Plautia stali* Vg3, BAA88077; *Riptortus clavatus*, AAB72001; *Samia cynthia pryri*, BAD91196; *Samia cynthia ricini*, BAB32641; *Saturnia japonica*, BAD91195; *Solenopsis invicta* Vg1 AAP47155; *Solenopsis invicta* Vg2 AAY22960; *Solenopsis invicta* Vg3 AAY22961 and *Tenebrio molitor*, AY714212.

<i>A. pernyi</i>	DGQRF-VVLASE-NRQSARGICGSMGEPRDD / CITTTLPAQCS-HCRGEDYKIQAAQVTCRPKIDQQFRSHRDHIKQGNP--EVTG--VPKVKQFKVPTACNA----
<i>A. yamamai</i>	DGQRF-VVLASE-NRQSARGICGSMGEPRDD / CITTTLPAQCS-HCRGEDYKIQAAQVTCRPKIDQQFRSHRDHIKQGNP--EVTG--VPKVIQFKVPTACNA----
<i>S. japonica</i>	DGQRL-IVLASE-NRQSARGICGSITGEPRDD / CITTTLVQCS-HCTVMEYRIQATQVTCRPKLDQQFRSYRDQIKQGGF--VVTG--VPKVKQFKVPAICNA----
<i>S. c. pryeri</i>	DGQRL-VVLASE-NRESARGICGSMGEPRDD / CITTTLPAQCS-HCRGEEYKIQAAQVTCRPKLDQQFRSHRDHIKQGNP--VVSQ--VPKVKQFKVPTSCNA----
<i>S. c. ricini</i>	DGQRL-VVLASE-NRESARGICGSMGEPRDD / CITTTLPAQCS-HCRGEEYKIQAAQVTCRPKLDQQFRSHRDHIKQGNP--VVSQ--VPKVKQFKVPTSCNA----
<i>B. mandarina</i>	DGQRF-VVFTQD-YRNSTRGICGRMSGEQRDD / CITTSRVPSQCS-HCRAGDYKIQHVQVTCRSKLDHDFRMYKEQIKKGNP--EVSQ--IPSVKQFKVPTVTCQF----
<i>B. mori</i>	DGQRF-VVFTQD-YRNSTRGICGRMSGEQRDD / CITTSRVPSQCS-HCRAGDYKIQHVQVTCRSKLDHDFRMYKEQIKKGNP--EVSQ--IPSVKQFKVPTVTCQF----
<i>L. dispar</i>	DGKRG-IFTTSQ-YRNITRGICGNSGDPLDD / CITTTPISAQCS-HCHSSNYQVQAVQVTCRSKLDHDFRMYKQDQHQGNP--QVTG--VPKVEQYRVPPTCTE----
<i>A. mellifera</i>	DGERVMLKAS-EDYRYSVRGLCGNFDHDSND / CFTMRPVVCS-GCTAVETKSKPKYKFCMEKN-EAAMKLKRIKEGANEDLSQKPV--STTEELTPVFCVKA----
<i>S. invicta</i> Vg1	DGEHILLMIS-DNYLNAVRLCGNYDTQPNND / -----
<i>S. invicta</i> Vg2	DGKRNVISAS-DAYRNAVRGLCGNFDSPNND / CFTTTPLPACRQ-GARPTERYPKKADLYCMPRN-DQSLDLKRRVEDGANEDTRKSVS--RMQVFQVPVSCSAA----
<i>S. invicta</i> Vg3	DGKHVRLYANGATYRNAIRGLCGNYDSRRDND / CFTTTPVPQCQLQ-DTRPVERVPKKYDLYCLSKN-NESMDLKRVEEGAKNEDPTQKPVN--KIQNFQIPVSCSAA----
<i>E. formosa</i>	DGSRRTKVG-NRYRDSVRGLCGNNDGESVDD / CFSKRFPVPSQCS-RCSEAEIRNKKIEFFCPVKS-DASEKVADRVEKGANEDLTQKNT--SKTEFKIPVSCSAA----
<i>P. nipponica</i>	DGQRVQIKAS-GKYRSDIRGLCGNFDGEPDND / CFSMRPLPCAAP-RFKPADTLKKIKFHLTKG-PTASHWLKMWKGVNEDFSKRS--HKQLEVDIPACVRH----
<i>A. rosae</i>	DGERIKLILN-NEYRDQIRGLCGTFNGEPATD / CFSLRPVATCA-GCKATRKQETTVKMHQVQSS-SSSQQLVHRKQGANEDFSQKSH--DKSSRIEVPESCVAQQ----
<i>B. germanica</i>	DGSRIMIQAS-NMYRNFTKGLCGNMDGEFVND / CVSEKPLDTMPLICHATESYITDVFVCPVLG-PAANYHMKLVKKGITPDLSSNRNGKRVVLVEIPIQCEPVLN--
<i>L. maderae</i>	ENQRFLLQAS-NIYRNKTRGLCGNMDGEITD / CFSAEPLKTCID-NSRAADTRIQQQFICLPDS-PASEHYLKLKKGINEDTRKKN--FVQLEVKIPTRKIKSH---
<i>P. americana</i> Vg1	DGKRVLQAS-NGYRDEVRGLCGTFDGEPTTD / CFSMRPLPCAAP-RFKPADTLKKIKFHLTKG-PTASHWLKMWKGVNEDFSKRS--HKQLEVDIPACVRH----
<i>P. americana</i> Vg2	DGERVMLHAG-NHYRNQVRGLCGTFDGEPTD / CISKALTECAP-LCREESHTTTEAFVFPFG-PTADHYTKLVKRGVSEDFSRKTD--IVNLRTVIPSRCVSKI---
<i>G. nigrofusca</i>	DGSRVQLQAD-SGYMSKTHGLCGPYNGEYID / CFSAPQNVCS-KCQATEIQQEVFPFCVENE-KARDFIQVPMGRVPEKLNQYQ--SEKLIVGVPOSCIFSY---
<i>P. stali</i> Vg1	DGKRVLVVS-NHHRDRVRGLCGTMDGEPSYD / CFSLRPVQSNP-NCKPTKNIQKHIEFFCRPDNDNLTHGWLRMVQKGANEDLSQKSS--NSKMSVMLEGCTSIH---
<i>P. stali</i> Vg2	DGHRAATMP-NSYRNKLRGLCGTFDGEPTD / CFSTEPQAVCS-NCKPSRKVERSIEFFCAPSTDSLTVHWNLVKKGANEDFSQKST--TKRMNVLPETCKPEKYN--
<i>P. stali</i> Vg3	DGQRVQIQIP-NSYRNMRVGLCGTFDGEPTD / CFSIRPQVTCN-NCQAGSLEKTIFFHLNES-SATRHWTVMKKGANEDFSQKSS--NYQMSVGLPEKCPNSYQM--
<i>R. clavatus</i>	DGARVQLSAS-SSYKGNVRGLAGTFDGEQAND / CFSTTPQPCAAS-HCRPTGKIQIVDFHCVQAS-SSSRNWAQMIKNGANEDFSAKER--HRQILLQLPTCCVPKA---
<i>A. aegypti</i> VgA1	DGYRA-RFFSDYSFYNNFVGLCGTNNGEYFD / CFSLRPLPVCS-KCAATEKISKYFDVHCFEKDSTQAKKYKSEIGRGYF--DFKSAPHKTYKFNYPKSCVYKAY--
<i>A. aegypti</i> VgB	DGYRA-RFFSDYSFYNNFVGLCGTNDGEYFSD / CFTLRPLPACS-KCVATDKVSKYFDVHCFEKSSPQAEKYKSEIARGYF--DFKSYASHKTFKFNYPKSCVYKAY--
<i>A. aegypti</i> VgC	DGYRA-RFFADQSYNNFVGLCGTNNGEYDD / CFSRPLPACS-HCAADTKPKYFDVHCRSADPAQALYKSKYQIKGYF--DMKSRSVKTKVFNIPKTCVHSQ--
<i>A. gambiae</i> (G3)	DGYRA-RFFADQSYNNFVGLCGTNNGEDED / CFTSRPLPACS-QCKATEKAPKYVDVHCRDATDSVAQLYKQIRKGVN--DMSNKSVTKTVKFFLPKCKVHVY---
<i>A. gambiae</i> (PEST)	DGYRA-RFFADQSYNNFVGLCGTNNGEDED / CFTNRALPACS-QCKAFEKIQYVDVHCRDATDSAAQLFKQIRKGVN--DMSNKSVTKTVKFFFPKCKVFAN---
<i>A. grandis</i>	DGQRVKVTATGNKLKRDVYGLCGRFSQDKHED / CFTIRPLPVNT--SVKQVVT-KNPVHFIQG-TKTAYYKSLIDQGGNEDFSRKSE--TRTARMEVAACN-----
<i>T. molitor</i>	DGQTVKLTAVNSKFRDASRGLCGTFDGEQETD / CFTLRPLPLKDGCAPRATVS-KQVDVYCASRSMRSGVGMWNEILKGASEDFSEQTA--SRKVRMEVPQSCMPRH---

Figure 2.

C-terminal alignment across all 31 available insect Vgs as identified in the legend to Fig. 1. Note that the *S. invicta* Vg1 sequence appears to be C-terminally truncated. Protein sequences are arranged in six blocks that represent the Lepidoptera, Hymenoptera, Orthoptera, Hemiptera, Diptera and Coleoptera, respectively. The alignment shows the conserved DGXR and GL/ICG motifs and identical aspartic acid (D), cysteine (C) and proline (P) residues in reverse highlight. With one exception, the DGXR and GL/ICG motifs are separated by 17 residues in the Lepidoptera, 19 residues in the Coleoptera and 18 residues in all other insects. For clarity of presentation, only the most proximal and distal parts of the C-terminus are shown, with the sequence break identified by a forward slash. For orientation purposes, the residue immediately preceding the break in *T. molitor* is D_[1660] and that immediately following the break is C_[1750].

Figure 3.

Southern blot analysis of pooled genomic DNA from *T. molitor* larvae. Ten micrograms of genomic DNA was digested with either *Xmn*I (X), *Bam*HI (B), *Sa*I (S) or *Hind*III (H), separated on 1% agarose and transferred onto Hybond-N+ membrane. The membrane was probed with a 666 bp fragment corresponding to nucleotides 2129–2794 of the Vg cDNA, washed at high stringency ($1 \times$ SSC; 0.1% SDS; 68 °C) and exposed to a phosphor screen for 40 h. Positively hybridizing fragments (arrows) were calibrated against the Generuler™ 1 kb Ladder (M).

Figure 4.

Profile of Vg mRNA abundance at various life stages of *T. molitor*. (A) Northern blot analysis of total RNA from larvae (L), male pupae (MP), female pupae (FP), fat body tissue from non-infected females at various days post-emergence (F1–F15) and fat body tissue from males at 6 days post-emergence (M6). Ten micrograms of total RNA was fractionated on denaturing 1% agarose-formaldehyde gels and transferred onto Hybond-N+ membrane. The membrane was probed with a 3' Vg cDNA fragment (upper panel) and 18S internal control (lower panel). (B) Relative abundance of Vg mRNA following normalization to the 18S internal control for the life stages described above. For F4–F15, Vg mRNA abundance was determined from the fat bodies of six individuals and for other samples, fat bodies from two individuals were used. Bars represent the mean $\pm$ standard error.

Figure 5.

Effect of *Hymenolepis diminuta* infection in intact females or fat-body culture with live *H. diminuta* parasites on Vg mRNA abundance in *T. molitor*. (A) Relative abundance of Vg mRNA for non-infected (open bars) and infected (hatched bars) *T. molitor* at various days post-emergence. Vg mRNA abundance was determined from the fat bodies of four individuals and significant differences between control and infected beetles are identified with an asterisk. (B) Relative Vg mRNA abundance in fat body tissue from non-infected *T. molitor* incubated *in vitro* with either 10 stage I–II parasites, 10 stage V–VI parasites or incubation medium alone (control). Vg mRNA abundance was determined independently from the fat bodies of six individuals and the bars represent the mean abundance $\pm$ standard error. Significant differences are represented by different letters, such that a vs. b corresponds to a probability of $P < 0.05$.