Isolation and characterization of a *Cotesia rubecula* bracovirus gene expressed in the lepidopteran *Pieris rapae*

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Polydnaviruses are endogenous particles that are crucial for the survival of endoparasitoid wasps, providing active suppression of the immune function of the lepidopteran host in which wasp larvae develop. The *Cotesia rubecula* bracovirus (CrBV) is unique in that only four gene products are detected in larval host (*Pieris rapae*) tissues and expression of CrBV genes is transient, occurring between 4 and 12 h post-parasitization. Two of the four genes, CrV1 and CrV3, have been characterized. CrV1 is a secreted glycoprotein that has been implicated in depolymerization of the actin cytoskeleton of host haemocytes, leading to haemocyte inactivation; CrV3 is a multimeric C-type lectin that shares homology with insect immune lectins. Here, a third CrBV-specific gene is described, CrV2, which is expressed in larval *P. rapae* tissues. CrV2, which is transcribed in haemocytes and fat body cells, has an ORF of 963 bp that produces a glycoprotein of approximately 40 kDa. CrV2 is secreted into haemolymph and appears to be internalized by host haemocytes. CrV2 has a coiled-coil region predicted at its C-terminus, which may be involved in the formation of putative CrV2 trimers that are detected in haemolymph of parasitized host larvae.

INTRODUCTION

Endogenous polydnaviruses (PDVs) are produced exclusively within the ovaries of certain hymenopteran endoparasitoids that belong to the ichneumonoid families Braconidae and Ichneumonidae (Stoltz & Vinson, 1979). Based on differences in host range and morphology, PDVs are subdivided into bracoviruses and ichnoviruses (Stoltz & Whitfield, 1992). Particles may contain one or several double-stranded circular DNA molecules (or segments) and are injected into the haemocoel of a host at oviposition, together with the parasitoid egg and other maternal protein secretions. Most of the proteins encoded by encapsidated genes are expressed within the parasitized host and function to suppress the host immune response [reviewed by Webb (1998); Kroemer & Webb (2004)]. Polydnaviruses are also known to interfere with the host endocrine system, causing developmental arrest and other physiological disorders [reviewed by Beckage (1998); Beckage & Gelman (2004)]. The PDVs are anomalous in that encapsidated circular DNA segments do not appear to encode PDV structural or replicative proteins, and new PDV particles or DNA are not produced in the parasitized host’s cells (Stoltz & Vinson, 1979). Instead, particle-associated DNA segments are known to originate from wasp chromosomal DNA and are transferred from one generation to the next, in their integrated form, in Mendelian fashion (Stoltz et al., 1986). Particles enter many host cell types, including haemocytes and fat body cells (Strand et al., 1992; Harwood & Beckage, 1994), and virus transcripts are detected in the first few hours following parasitization. Transcripts are generated either transiently (Asgari et al., 1996) or persistently (Strand et al., 1992) during parasitism.

The *Cotesia rubecula* bracovirus (CrBV) is unique as it appears to generate only four gene products, production of which in the larval host (*Pieris rapae*) is highly transient, from 4 to 12 h post-parasitization (h.p.p.) (Asgari et al., 1996). By contrast, the *Campoletis sonorensis* ichnovirus (CsIV) is estimated to express over 35 genes that belong to several gene families (Turnbull & Webb, 2002) and expression of at least some genes continues throughout development of the parasitoid larva (Webb, 1998). Transient gene expression is associated with reversible inactivation of the cellular immune system, which is considered to be an advanced evolutionary adaptation that restores the
parasitized host’s ability to defend the developing parasitoid against pathogens or other parasitoids.

Asgari et al. (1996, 1997) cloned and sequenced CrV1-encoding DNA from CrBV, demonstrating that CrV1 is an encapsidated gene that is expressed as a single transcript in parasitized host haemocytes. CrV1 is secreted from infected cells into serum interacting with the surface of haemocytes (Asgari et al., 1997). Although the exact mode of action is unknown, the presence of depolymerized actin in CrV1-treated haemocytes suggests that CrV1 interaction with the cell surface leads to depolymerization of cytoplasmic actin structural components (Asgari et al., 1997). Without functional actin filaments, haemocytes are unable to undergo the rearrangements of the cytoskeleton that are required for immune-related spreading and phagocytosis reactions (Rosales et al., 1994; Yano et al., 1994; Strand & Pech, 1995; Asgari et al., 1997). Homologues of CrV1 have been found to occur in six Cotesia species and the matching phylogenetic trees created by analysis of wasp 16S rRNA and NADH1 genes also matched that produced by analysis of CrV1 homologue sequences (Whitfield, 2000), suggesting that PDVs were not acquired independently among Cotesia species, but co-evolved with the hymenopteran parasitoid (Whitfield & Asgari, 2003).

More recently, a second CrBV gene (CrV3) was characterized as a C-type lectin (CTL) (Glatz et al., 2003). CrV3 has homologues in baculoviruses that are associated with Cotesia ruficrus and Cotesia karyai (Teramoto & Tanaka, 2003) and this group of CTLs forms a unique CTL family. Interestingly, the CrV3 homologues are related more closely to invertebrate CTLs, which have been implicated in humoral immune defence of such animals (Haq et al., 1996; Saito et al., 1997; Arai et al., 1998; Kakiuchi et al., 2002; Yu & Kanost, 2003), than to known viral lectins. CrV3 forms multimeric structures in the haemolymph, composed of monomers (occurring as two glycoforms for CrV3) that each contain a single carbohydrate-binding domain, features that are shared with invertebrate immune CTLs (Kilpatrick, 2002; Glatz et al., 2003).

In this study, we report the isolation and characterization of CrV2, the third of four expressed CrBV genes to be isolated. Like CrV1, CrV2 has a coiled-coil domain and is found in oligomeric form in the haemolymph of parasitized larvae, where it is taken up by host haemocytes.

**METHODS**

**Insect cultures.** The parasitoid C. rubecula and its host were maintained at 25 °C on a 14:10 h (light:dark) photoperiod. P. rapae larvae were reared on cabbage plants. Adult C. rubecula wasps were fed with honey–water solution.

**CrBV and genomic DNA isolation.** Virus purification and genomic DNA extraction were carried out as described previously (Glatz et al., 2003).

**Southern and Northern blot hybridization.** DNA samples were run on a 1% agarose gel and transferred to a nylon membrane as described by Sambrook et al. (1989). RNA was isolated from unparasitized and 6 h-parasitized P. rapae caterpillars according to Chomczynski & Sacchi (1987). RNA samples were run on 1% agarose gels under denaturing conditions using formaldehyde and transferred to nylon membranes as described by Sambrook et al. (1989). Probes were prepared as described by the manufacturer (Ready-To-Go DNA labelling beads; Amersham Biosciences). Slot-blots were carried out by using a Bio-Dot SF microfiltration apparatus (Bio-Rad), according to the manufacturer’s instructions.

**5’ amplification of CrV2 cDNA (5’ RACE).** Partial CrV2 cDNA was extended in the 5’ direction by using the 5’ RACE system for rapid amplification of cDNA ends (Life Technologies). PCR product obtained from 5’ RACE was ligated into the pGEM-T Easy vector as described by the manufacturer (Promega). The insert was sequenced by using M13 forward and reverse primers.

**Computer analysis.** Sequences were compared against those contained in GenBank by using a nucleotide BLAST search, accessed via the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov/blast). All CrV2 protein analysis tools were accessed through the ExPASy molecular biology server (http://us.expasy.org/tools).

**RT-PCR.** Primers specific to the CrV2 ORF (Fig. 1) were used in RT-PCR analysis of RNA from unparasitized and 6 h-parasitized P. rapae larvae by using AMV reverse transcriptase (AMV-RT; Promega). XhoI and HindIII restriction sites were added to primer sequences (underlined) to provide sites for direct ligation of the fragment into the pQE30 expression vector (Qiagen). Primer CrV2-F (5’-GCATGCCCGTTGCAAGACAGAAG-3’) was used in the RT reaction, followed by PCR using primers CrV2-R and CrV2-F (5’-GCAGGCTATGCCTTTGCAAGACAGAAG-3’).

**Collection of protein samples and Western blotting.** Haemolymph, haemocyte and fat body cells were collected and prepared as described previously (Glatz et al., 2003). Protein samples were electrophoresed on denaturing 12% SDS–polyacrylamide gels, as described by Laemmli (1970). Standard conditions were denaturing; however, non-denaturing conditions were sometimes used, whereby SDS was absent from gels, sample and running buffers. Proteins were either stained within gels by using Coomassie brilliant blue (Sigma) or transferred to a nitrocellulose membrane as
Cotesia rubecula bracovirus gene expression
described by Sambrook et al. (1989). Before obtaining anti-CrV2, blots were probed with either a 1:10,000 dilution of peroxidase-conjugated Helix pomatia lectin (HPL, 50 ng ml⁻¹; Sigma) or an anti-polyhistidine mAb (clone His-1; Sigma).

Expression and purification of bacterial CrV2. Primers CrV2-F and CrV2-R were designed to amplify the ORF of the CrV2 gene, excluding a putative signal sequence corresponding to the first 20 aa of the protein (see Fig. 1 and RT-PCR for CrV2). These primers were used in PCR of pGEM-T Easy vector (Promega) containing the CrV2 ORF to obtain the required fragment for ligation to the pQE30 bacterial expression vector (Qiagen). Production of bacterial CrV2 (containing six additional, vector-derived histidine residues) was induced by addition of 1 mM IPTG to the bacterial suspension for 2 h at 37 °C. The resulting fusion protein was largely contained in the insoluble fraction and was purified under denaturing conditions as described by the manufacturer (Qiagen). Samples were dialysed overnight against TBS (0.15 M NaCl, 0.01 M Tris, pH 8.0) at 4 °C.

Anti-CrV2 polyclonal antibody production. Purified bacterial CrV2 was visualized on preparative 12% SDS–acrylamide gels by staining with water-dissolved Coomassie brilliant blue (Sigma). CrV2 protein bands were excised from the gel and used for immunization of two rabbits as described previously (Glatz et al., 2003). The antiserum was used at a dilution of 1:5000. Bound anti-CrV2 was then visualized by alkaline phosphatase-labelled secondary anti-rabbit antibody (1:10,000).

Fluorescent labelling of CrV2 associated with P. rapae haemocytes. Unparasitized and 24 h-parasitized larvae were bled into PBS that was saturated with phenylthiourea before centrifugation at 2300 g for 5 min. The pellet was then resuspended gently in PBS before transfer to multiwell slides. Time was allowed for settling before fixing cells with 4% paraformaldehyde in PBS before transfer to multiwell slides. Time was allowed for settling before fixing cells with 4% paraformaldehyde in PBS. Anti-CrV2 antiserum and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody were used to visualize CrV2 associated with haemocytes, as described previously (Asgari et al., 1996).

RESULTS
CrBV genes are expressed transiently within a few hours of infection of host tissues (Asgari et al., 1996). When total CrBV genomic DNA was used to probe total RNA from parasitized P. rapae caterpillars, four abundant virus transcripts were detected 4 h.p.p. (Asgari et al., 1996). The resulting signal became stronger at 6 h, to the extent that it was overexpressed compared with the amount of transcript at 4 and 12 h. Although additional virus transcripts may be expressed at low levels that are masked by the strong signals at 6 h after parasitization, the four detected transcripts are nevertheless referred to as CrV1–4 by decreasing size. CrV1 and CrV3 were previously isolated and identified by screening a cDNA library made from 6 h-parasitized larvae by using total CrBV DNA as a probe (Asgari et al., 1996; Glatz et al., 2003). A similar approach was employed here to isolate the CrBV gene encoding CrV2 and to investigate its possible role in the host–parasitoid interaction.

Isolation and characterization of CrV2
Several screens of the cDNA library led to the isolation of an approximately 450 bp cDNA (CloneC) that encoded part of a putative CrBV gene and included a poly(A) tail (Fig. 1). The fragment was cloned and sequenced, allowing primers to be designed to amplify 290 bp of the 5’ end (CloneC-F and CloneC-R; Fig. 1). To confirm the cDNA fragment as particle-derived, the amplified fragment was then used as a probe in both a Southern blot of CrBV DNA (Fig. 2a) and a Northern blot of RNA from naïve and 6 h-parasitized larvae (Fig. 2b). Hybridization occurred to a CrBV restriction fragment of >15 kbp and to a parasitism-specific transcript of approximately 1-2 kbp. These data, and the fact that the same probe bound to genomic DNA from female wasps but not to genomic DNA from P. rapae (data not shown), indicate that the cDNA originated from particles that were introduced to the larvae at oviposition. Based on the transcript size being intermediate between CrV1 (1-4 kbp) and CrV3 (1-1 kbp), matching the transcript size of CrV2-encoding mRNA, we designated the newly isolated gene as CrV2. Binding of the cDNA to a single RNA band on the Northern blot revealed that CrV2 shows no significant homology to other CrBV-related genes within the 290 bp probe. Slot-blot analysis of RNA isolated from haemocytes and fat body cells from 6 h-parasitized larvae showed that there was no significant difference in the amount of CrV2 transcripts detected in the two samples (Fig. 2d). In order to measure RNA loading within slots, a fragment of 18S rRNA was used from P. rapae as a control.

5’ RACE was employed to extend the cDNA at the 5’ end and complete the ORF. This method produced a fragment of approximately 850 bp (data not shown), which was subsequently sequenced. Sequence data from the 5’ RACE fragment were combined with those from the 450 bp cDNA clone to assemble the total CrV2 ORF of 960 bp and flanking sequences (Fig. 1). A methionine codon (ATG) at the beginning of the ORF was identified as the only possible codon with a suitable nucleotide sequence environment for functional initiation (Cavener & Ray, 1991). Computer analyses of the deduced amino acid sequence revealed a putative signal peptide encompassing the first 20 aa of the protein, with a cleavage point predicted at the end of the signal sequence (Fig. 1), indicating that the CrV2 protein is probably secreted from infected cells.

Four putative N- and six putative O-glycosylation sites were predicted in the ORF, as well as a polyadenylation signal approximately 120 bp downstream of the stop codon (Fig. 1). These data were used to generate primers specific to the CrV2 ORF (CrV2-F and CrV2-R; Fig. 1). Comparison of RT-PCR and genomic DNA PCR products that were generated by utilizing these primers revealed no sequence differences, indicating that no introns are present in the genomic CrV2 DNA. The CrV2 nucleotide and deduced amino acid sequences were compared against known sequences in GenBank; however, no significant homology was detected.

CrV2, without the signal peptide, was expressed in Escherichia coli and produced an approximately 40 kDa fusion protein, following induction with IPTG (Fig. 2c). Computer analyses predicted a molecular mass of 33-7 kDa and a pl of...
8.94 for secreted CrV2. Purified recombinant CrV2 was used to immunize rabbits for production of polyclonal anti-CrV2 antibodies. Western blot analysis of serum from non-parasitized and 6 h-parasitized *P. rapae* larvae, probed with anti-CrV2, allowed visualization of the 37 kDa CrV2 only in parasitized larvae (Fig. 3a). Previous data showed the presence of a parasitism-specific glycoprotein in the haemolymph of *P. rapae* larvae, the production of which was initiated at approximately 6 h.p.p. (Asgari, 1997; Fig. 3b). By using anti-CrV2 to probe serum proteins from 6 h-parasitized larvae, it was determined that the previously unidentified glycoprotein is CrV2 (Fig. 3b). These data confirm that CrV2 is a secreted glycoprotein and, further, that it contains N-acetyl-D-galactosamine residues at its O-glycosylation sites, as it was previously detected by GalNAc-specific HPL (Asgari, 1997). Western blot analyses of larval serum, haemocytes and fat body cells at various points after parasitization showed that CrV2 was present in each sample at 6 h.p.p., reached a maximum level at about 24 h.p.p. and was declining at 48 h.p.p. (Fig. 3c). These data are consistent with secretion of CrV2 into cell-free haemolymph from CrBV-infected haemocytes and fat body cells.

**CrV2 forms trimers**

When cell-free haemolymph proteins were analysed under non-denaturing conditions, a putative CrV2 trimer of approximately 98 kDa was detected in parasitized larvae (Fig. 4a). Coiled-coil regions are commonly associated with the multimerization of proteins (Alber, 1992). COILS software predicted the presence of a coiled-coil region near the C-terminus of CrV2, comprised of aa 266–313 (Fig. 4b). Analysis of the coiled-coil region with MULTICOIL software predicted a high probability of trimer formation, due to the presence of the C-terminal coiled-coil region (data not shown).

**Relative expression of CrV2 and CrV3**

During an earlier CrV3-related study, anecdotal evidence pointed to large differences in the amount of CrV2 and CrV3 transcripts and protein detected in parasitized larvae. Previous data also indicated that CrV3 transcript levels are generally lower than transcript levels from the other three major CrBV genes (Asgari *et al.*, 1996). Therefore, several experiments were undertaken in order to directly compare relative levels of transcription and protein production in
cell-free haemolymph, haemocytes and fat bodies of parasitized larvae. Previous work showed that each of the four CrBV genes expressed in parasitized larvae was expressed transiently (with maximum expression near 6 h.p.p.) and that the up- and downregulation of each gene occurred over similar periods (Asgari et al., 1996). Thus, 6 h-parasitized larvae were used for the comparison. Slot-blot analysis was used to compare relative CrV2 and CrV3 transcript levels contained in RNA extracted from naïve and parasitized P. rapae larvae (Fig. 5a). Probing similar amounts of RNA with CrV2 and CrV3 probes indicated that CrV2 transcript made up a significantly greater proportion of total transcripts than CrV3 transcript. Indeed, RNA probed with 32P-labelled CrV2 produced a visible signal in just a few hours of autoradiograph exposure, whereas the same amount of RNA probed with 32P-labelled CrV3 required nearly 3 days

**Fig. 3.** Expression and secretion of CrV2. The position of CrV2 is indicated by an arrowhead in each blot. (a) Western blot analysis (12% SDS-PAGE; anti-CrV2 antiserum, 1:5000) of cell-free haemolymph from non-parasitized (N) and 6 h-parasitized (P) P. rapae larvae. (b) A parasitism-specific glycoprotein (arrowhead) detected at 6 h.p.p. on Western blots containing proteins extracted from serum from naïve (N) and parasitized P. rapae larvae and probed with a GalNAc-specific HPL (1:10 000). Anti-CrV2 antibodies (1:5000) confirmed that the secreted glycoprotein is CrV2 (P6). (c) Western blot analyses (12% SDS-PAGE; anti-CrV2 antiserum, 1:5000) of P. rapae cell-free haemolymph, haemocytes and fat body cells. Proteins were collected at various time points (h) after parasitization and are indicated above the blots. Total haemocytes or fat body cells were pooled from three larvae at each time point, from which 20 μl was loaded per lane. Similarly, for haemolymph samples, 5 μl cell-free haemolymph from each pool was loaded per lane. Protein source is shown below blots. For each protein source, expression is detected at 6 h.p.p., large amounts of CrV2 are still present at 24 h.p.p. and CrV2 levels are in decline at 48 h.p.p.
autoradiograph exposure to produce a visible signal (Fig. 5a).

Western blot analyses of similar amounts of protein from haemocytes, serum and fat bodies, probed with both anti-CrV2 and anti-CrV3, indicated that this difference is maintained at the protein level (Fig. 5b). In the comparative analyses shown, CrV2 was detected in large amounts in each sample, while CrV3 was not detected under conditions of the same amount of total loaded protein (Fig. 5b). These data indicate that CrV2 protein is present in significantly higher amounts than CrV3 in haemocytes, cell-free haemolymph and fat bodies of parasitized larvae. As this reflects the relative amounts of transcripts, this difference is regulated at the transcriptional level. The biological significance of this differential expression is not known. The difference may reflect a function different from that of CrV3, in that CrV2 (like CrV1) targets haemocytes, which internalize the protein in a systemic fashion and therefore require large amounts of active protein in the serum. In contrast, CrV3 appears to function in cell-free haemolymph and may possibly be recycled after binding to a target molecule. Alternatively, the target molecule of CrV3 may be present in small amounts, e.g. an immune-induced form of a molecule that contains an unusual glycosylation or proteolytic cleavage site.

**Immunofluorescence detection of CrV2 in haemocytes**

Most of the PDV genes characterized so far target haemocytes of parasitized larvae, e.g. CrV1 (Asgari et al., 1996, 1997), EP1 from *Cotesia congregata* bracovirus (CcBV) (Beckage & Kanost, 1993; Beckage et al., 1994), VHv1.1 from *Campoletis sonorensis* ichnovirus (Dib-Hajj et al., 1993) and EGF-like gene products from *Microplitis demolitor* bracovirus (Strand et al., 1997; Trudeau et al., 2000). To investigate whether haemocytes are targeted by CrV2, haemocytes were isolated from larvae at different times post-parasitization and tested for CrV2 presence by staining with FITC-linked secondary antibody. The maximum amount of staining occurred at 24 h.p.p. (Fig. 6a). At this point, much of the CrV2 appeared to be localized within the haemocytes in large endosomes (Fig. 6b). Most CrV2 protein is found in cell-free haemolymph at this stage, which suggests that CrV2 is taken up by haemocytes, similarly to CrV1. CrBV-related proteins remain present in the haemolymph for several days after parasitization, although transcripts are produced only transiently (Asgari et al., 1996). However, a low, persistent level of CrBV expression that was not detected by Northern blotting cannot be ruled out. A comprehensive, real-time, RT-PCR quantification approach is required to confirm expression levels.

**DISCUSSION**

The relatively small number of four CrBV genes that are expressed in the host provides the opportunity to elucidate the role of CrBV in parasitization in a comprehensive manner, compared with other PDV systems where a large number of genes are expressed by PDV-infected host cells (Kroemer & Webb, 2004). The overall role of PDVs that encode active immune suppressors is widely accepted.
Nearly all of the characterized genes that have been reported so far are known to target host haemocytes; however, other functions, such as alteration of developmental regulation and behaviour, are also known (Beckage & Gelman, 2004). From our investigations, it appears that the main role of CrBV is immune suppression of the larval host haemocytes. CrV1 is known to be taken up by host haemocytes, causing inactivation of F-actin and reducing the ability of infected haemocytes to carry out immune-related tasks, such as phagocytosis and spreading (Asgari et al., 1996, 1997). Thus, CrV1 acts as a haemocyte-specific toxin, although its transient expression means that cells recover after a few days. Interestingly, a CrV1 homologue was identified in CcBV, but differed in that expression was strong for 72 h and continued at low levels throughout development of the wasp larvae (Le et al., 2003). It is believed that the CrV1 homologue affects haemocytes by mediating apoptosis (unlike for CrBV), perhaps a function of continuous expression. However, the level of complexity of the CcBV system precludes linking apoptosis to a single gene for the time being.

The function of CrV2 is, as yet, undetermined. The large amount of CrV2 in haemolymph and haemocytes at 24 h.p.p., and low levels of CrV2 transcripts in haemocytes at the same time point, indicate that haemocytes internalize the protein. In such a scenario, haemocytes and fat body cells would secrete CrV2 into the serum, from where haemocytes acquire CrV2, as is the case for CrV1 (Asgari et al., 1996, 1997). Further studies are required to determine the molecular interactions between CrV2 and haemocytes in vitro, in both the presence and absence of other CrBV proteins. Injection of active recombinant CrV2 into naïve larvae may also reveal the effects of CrV2 on haemocytes and whether P. rapae haemolymph proteins are required for haemocyte uptake. As CrV2 is similar to CrV1 in terms of monomer size, expression levels, presence of a coiled-coil region and formation of small oligomers, together with the fact that most characterized class II PDV genes (those expressed in the host caterpillar; Theilmann & Summers, 1988) appear to target haemocytes, may further indicate that the function of CrV2 is similar to CrV1. It is possible that CrV2 enhances or complements the activity of CrV1 by targeting a distinct haemocyte type.

CrV3 is also implicated in immune disruption in that it is related closely to invertebrate immune proteins. Apart from CrV3 homologues that are expressed by other Cotesia-associated bracoviruses (Teramoto & Tanaka, 2003), the

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Fig. 5. Transcription of CrV2. (a) Northern slot-blot comparing relative levels of CrV2 and CrV3 transcripts in total RNA from un- and 6 h-parasitized P. rapae larvae. Probes consisted of 32P-labelled CrV2 and CrV3 fragments to measure virus transcripts and a 32P-labelled P. rapae 18S rRNA gene fragment. Putative amounts of loaded RNA are shown at the top; the source of RNA (naïve versus parasitized larvae) is shown at the bottom. Autoradiograph exposure period is shown on the left; probe type on the right. CrV2 transcripts comprise a significantly greater proportion of total transcripts than CrV3 transcripts. (b) Comparison of relative CrV2 and CrV3 protein levels in haemocytes (H), cell-free haemolymph (C) and fat body (F) from 6 h-parasitized P. rapae larvae. The amount of each protein sample was calibrated, allowing direct comparison of CrV2 and CrV3 levels within a given sample. The probe used for each analysis is shown beneath the blot. CrV2 appears to be expressed at a significantly higher level in each sample than CrV3.
closest relatives to CrV3 were insect CTLs that are secreted into cell-free haemolymph on induction by foreign elicitors, such as lipopolysaccharide on bacterial surfaces. These CTLs act as immune molecules by binding to specific sugar moieties associated with foreign surfaces, rendering them visible to the immune system and facilitating their removal from circulation. It seems probable that the unusual regulation of the CrV3 protein is associated with its role in immune suppression, as opposed to immune protection.

The *P. rapae*–CrBV–*C. rubecula* system represents a unique opportunity to develop a comprehensive model of immune-suppressive activity carried out by CrBV and thus to glean more general information relating to virus-related manipulation of host physiology. Genes such as CrV1 and CrV3, whose homologues occur in a range of *Cotesia*-related bracoviruses, also raise interesting questions about the origin of PDVs and their genes. Evolutionary studies will explore the origin of this relationship further by targeting the ancestral PDV forms and the way PDVs have apparently driven the successful radiation of certain ichneumonoid endoparasitoids.

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