A polydnavirus-encoded protein of an endoparasitoid wasp is an immune suppressor

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The molecular mechanism by which polydnaviruses of endoparasitoid wasps disrupt cell-mediated encapsulation reactions of host insects is largely unknown. Here we show that a polydnavirus-encoded protein, produced from baculovirus and plasmid expression vectors, prevents cell surface exposure of lectin-binding sites and microparticle formation during immune stimulation of haemocytes. The inactivation of immune-related cellular processes by this protein was analysed using a specific lectin and annexin V and shown to be virtually identical to polydnavirus-mediated effects on haemocytes. Cytochalasin D application has similar effects on haemocytes, suggesting that the immune suppression by the polydnavirus protein is caused by the destabilization of actin filaments. Since the exposure of cell surface glycoproteins and the formation of microparticles are part of an immune response to foreign objects or microorganisms and a prerequisite for cell-mediated encapsulation of microorganisms and parasites, the virus-encoded protein may become an important tool for the inactivation of cellular immune reactions in insects and an essential component in understanding immune suppression in parasitized host insects.

Introduction

Polydnaviruses of hymenopteran endoparasitoids enable parasitic wasps to escape the cellular defence reactions of their habitual host caterpillars. Due to the paucity of molecular knowledge concerning cellular immunity in insects, the exact mechanism of virus-mediated inactivation of host immune defences is difficult to investigate. Moreover, some endoparasitoid species use a complex combination of different wasp components to circumvent the host’s defence reactions. For example, the eggs of the ichneumonid Campoletis sonorensis are protected against the defence reaction of the host Heliothis virescens through the complex action of calyx fluid proteins (Summers & Dib-Hajj, 1995; Webb & Luckhart, 1996) and virus-encoded protein(s) (Edson et al., 1981; Li & Webb, 1994). The calyx fluid proteins are probably involved in the immediate inactivation of haemocytes, whereas the protection of the growing parasitoid at a later stage depends on the expression of the virus genome, including a group of genes encoding cysteine-rich proteins (Li & Webb, 1994). Similar complementary activities or synergistic effects of maternal ovarian fluid proteins and polydnaviruses are reported in other endoparasitoid systems (Kitano, 1986; Strand & Dover, 1991; Tanaka, 1987). The result of parasitization by many hymenopteran parasitoids is cellular alteration of host haemocytes, which correlates with a failure to aggregate or spread on a glass surface (Davies et al., 1987; Stoltz & Guzo, 1986; Strand, 1994). Although polydnavirus injection into caterpillars can induce haemocyte changes similar to those observed in parasitized caterpillars (Asgari et al., 1996; Beckage et al., 1994; Luckhart & Webb, 1996; Stoltz & Guzo, 1986; Summers & Dib-Hajj, 1995), the question remains whether the virus-coded proteins act in the cytoplasm of infected haemocytes or whether they are secreted into the haemolymph and interact with the surface of other haemocytes.

In the braconid Cotesia rubecula the newly deposited egg is protected against the defence reactions of the host Pieris rapae through a layer of calyx fluid glycoproteins which provide passive protection of the developing wasp during embryogenesis (Asgari & Schmidt, 1994). In the absence of Cotesia rubecula polydnavirus (CrV), the developing parasitoid is protected only until the larva emerges from the chorion, whereas in the presence of CrV the newly hatched larva is protected by a virus-mediated mechanism. The CrV infects host haemocytes and fat body cells, which subsequently express the virus genome in a short time period between 6 and 12 h after parasitization (Asgari et al., 1996). Among CrV
genes expressed in the host (CrV1 and CrV2), only CrV1 was detected in the haemocytes. A cDNA clone coding for CrV1 has been characterized by sequencing. The deduced open reading frame is predicted to code for a secreted product (Asgari et al., 1996).

Here we show that CrV1 interferes with normal haemocyte functions. CrV1 is secreted from infected haemocytes and fat body cells into the haemolymph, where it interacts with the surface of haemocytes. The result of CrV1 interaction is a transient inhibition of immune-related haemocyte-specific surface changes, involving the exposure of a lectin-binding protein and microparticle formation. A rearrangement of lectin-binding sites on the haemocyte surface has been suggested both in encapsulation of foreign tissues and in haemolymph coagulation (Rizki & Rizki, 1983; Theopold & Schmidt, 1997). Inhibition of haemocyte activation processes precludes cellular encapsulation reactions during a critical time when the parasitoid larva emerges from the chorion.

Methods

■ Insect cultures and parasitization. Parasitoid wasps, C. nubicula (Hymenoptera: Braconidae), were reared on P. rapae (Lepidoptera: Pieridae) grown on cabbage as described previously (Asgari & Schmidt, 1994).

■ Construction of a recombinant baculovirus. A full-length CrV1 cDNA cloned in pBluescriptKS(+) (Asgari et al., 1996) was digested with PstI and KpnI restriction enzymes to allow the complete cDNA to be directionally cloned into corresponding restriction sites in a transfer vector (pBacPAK8, Clontech). Baculovirus DNA (BacPAK6, Clontech) was also digested with Bsa I and then co-transfected with the constructed transfer vector into Spodoptera frugiperda cells (Sf21) using standard procedures (Summers & Smith, 1987). Recombinant baculoviruses were isolated by plaque assays and propagated as previously described (Summers & Smith, 1987).

■ Isolation, partial purification and injection of the recombinant protein. Sf21 cells were infected with parental and recombinant viruses. At 18 h after infection, the complete medium (Grace’s medium supplemented with 10% foetal bovine serum) was removed and cells were washed with serum-free medium. The cells were then incubated with serum-free medium for 4–5 h at 27 °C. The culture medium was collected and cells were removed by centrifugation at 2000 r.p.m. for 2 min. The supernatant was centrifuged at 35,000 r.p.m. (Beckman TL-100) for 1 h, to pellet any virus particles. The supernatant was concentrated ca. 10 times and desalted using a Centricon-10 centrifugal concentrator (Amicon). Equal amounts of recovered supernatant (ca. 15 µl), representing similar amounts of protein from recombinant and parental supernatants, were injected into fourth instar P. rapae caterpillars using a flame-extended Pasteur pipette (the concentration of CrV1 in the recombinant fraction was between 0·1 and 0·4 µg/µl). The larvae were bled 24 h later and haemocytes were examined.

■ Construction of recombinant plasmids and transformation of insect cells. The complete cDNA fragment coding for CrV1 in pBluescriptKS(+) was excised with BamHI and NotI, and re-cloned into corresponding sites in pELHR4 plasmid vector under control of an immediate early gene promoter of the Autographa californica nuclear polyhedrosis virus (Jarvis et al., 1990) using standard recombinant DNA techniques. Sf21 cells were transfected with recombinant plasmids as previously described (Jarvis et al., 1990; Summers & Smith, 1987). A control transfection was performed using the vector plasmid devoid of a DNA insert. The culture medium was collected from transformed cells 4 days after transfection and proteins were purified by affinity chromatography using cyanogen bromide-activated Sepharose beads (Sigma) coupled with Helix pomatia lectin (HPL) (according to the Affinity Chromatography Manual, Pharmacia). The column-bound proteins were eluted with a buffer containing N-acetylgalactosamine (GalNAc) at a concentration of 200 mM. The average concentration of recovered CrV1 was estimated to range between 0·1 and 0·5 µg/µl of the eluate, using HPL and Coomassie-labelling of electrophoretically separated glycoproteins of known concentration.

■ Protein analysis. Protein extracts were analysed on denaturing 12% SDS-polyacrylamide gels, as described by Laemmli (1970); SeeBlue pre-stained standard markers (Novex) were used to determine the molecular mass. The proteins were stained with Coomassie blue in the gel or transferred onto a nitrocellulose membrane (Amersham) as described by Sambrook et al. (1989). Western blots were probed with peroxidase-conjugated HPL (50 ng/ml, Sigma) (Theopold et al., 1996).

Antiserum against CrV1 was obtained using affinity-purified glycoproteins from CrV1-producing baculovirus-infected cell culture medium. Two rabbits were injected with column fractions containing ca. 4 µg of CrV1 mixed with Freund’s adjuvant followed by three subsequent boost injections at 3 week intervals. Antiserum or presernum was used in a dilution of 1:5000 on Western blots followed by alkaline phosphatase-conjugated secondary antibodies (dilution 1:10,000).

■ HPL staining of haemocytes. P. rapae haemocytes were labelled using fluorescein isothiocyanate (FITC)-conjugated HPL as previously described (Asgari et al., 1996). Briefly, caterpillars were bled into PBS (138 mM NaCl, 2·7 mM KCl, 1·47 mM KH₂PO₄, 7·3 mM Na₂HPO₄, pH 7·6) or PBS supplemented with 0·25 mM CaCl₂ on multi-well slides. Haemocytes were then allowed to settle and fixed with 4% paraformaldehyde in PBS. Cells were washed with PBS and incubated with 0·25% BSA in PBS followed by incubation with the lectin (10 µg/ml 0·25% BSA–PBS). They were then washed and examined under indirect UV illumination.

■ F-Actin staining of haemocytes. Haemocytes collected from caterpillars were stained for actin using FITC-conjugated phalloidin as previously described (Asgari et al., 1996).

■ Cell staining with annexin V. Pieris caterpillars were bled into a multi-well slide in PBS and allowed to settle for 10 min. The buffer was removed and cells gently washed with binding buffer (HEPES buffered saline solution supplemented with 0·25 mM CaCl₂). FITC-conjugated annexin V (10 µg/ml, R&D Systems) was diluted 1:2 in binding buffer, added to haemocytes and incubated for 10 min at room temperature. Cells were covered with a cover-slip and examined by microscope under indirect UV illumination.

Results

Haemocyte activation

Actin rearrangement has been found to occur in haemocytes from polydnavirus-infected caterpillars (Asgari et al., 1996; Webb & Luckhart, 1994). To determine whether changes in F-actin filaments are causally related to the observed virus-mediated haemocyte alterations, we searched for cellular haemocyte properties that are required for cell-mediated immune reactions. Since lectin-binding sites are altered in haemocytes, we stained for actin using FITC-conjugated phalloidin as previously described (Asgari et al., 1996).
Fig. 1. Effects of parasitization and cytochalasin D treatment on haemocytes. (A) Exposure of HPL-binding protein on the surface of activated haemocytes after treatment with FITC-conjugated HPL; (B) exposure of HPL-binding protein on the surface of haemocytes from a caterpillar parasitized 24 h before haemocyte isolation; (C) exposure of HPL-binding protein on the surface of haemocytes from a caterpillar injected with cytochalasin D (10 µM) 2 h before haemocyte isolation. Left, phase-contrast pictures; right, corresponding exposures with indirect UV light.

we investigated the functional significance of cell surface changes in haemocytes from parasitized and non-parasitized *P. rapae* caterpillars using HPL, which was previously shown to bind to haemomucin on *Drosophila* haemocytes (Theopold et al., 1996). When *P. rapae* haemocytes were isolated in calcium-containing buffer, the relative amount of HPL binding to granulocytes was initially low but increased continuously over a period of 1 h (Fig. 1A). When haemocytes were isolated in the presence of EDTA instead of calcium, no HPL binding was observed over the same period of time (not shown). This suggests that the HPL-binding sites emerged on the surface after haemocytes were bled in calcium-containing buffer and exposed to a glass surface. It is worth noting that HPL binding is calcium-independent (Hammarström & Kabat, 1971). No staining was observed on haemocytes from parasitized caterpillars (Fig. 1B). To test whether these changes were dependent
on functional actin filaments, cytochalasin D was injected into caterpillars and haemocytes were isolated in the presence of calcium. At 2 h after injection, haemocytes failed to bind HPL on the surface (Fig. 1C). This suggests that intact actin filaments are required for the exposure of HPL-binding sites on the surface of haemocytes. Since lectin-binding proteins are involved in immune-related processes, the exposure of HPL-binding sites in isolated haemocytes is probably part of the haemocyte activation process.

To test whether the cellular process of microparticle formation is inhibited in polydnavirus-infected caterpillars, isolated haemocytes from parasitized and non-parasitized caterpillars were analysed. When haemocytes are isolated in calcium-containing buffer and exposed to glass surfaces, blebbing and microparticle formation occur (Theopold & Schmidt, 1997). A specific property of microparticle formation is the exposure of phosphatidylserine (PS) on the outer leaflet of cellular and particle membranes, which is specifically recognized by annexin V (Dacharyprigent et al., 1996; Qu et al., 1996). We investigated the presence of exposed PS in haemocyte preparations using annexin V, and found that microparticle formation and PS exposure are features of normal haemocytes (Fig. 2A, B), but are virtually absent in haemocyte preparations from parasitized caterpillars (Fig. 2C, D).

This suggests that polydnavirus infection of the host insect interferes with the cellular activation of haemocytes. The observation that cytochalasin D causes similar effects suggests that haemocyte activation, including exposure of HPL-binding sites on the haemocyte surface and microparticle formation, requires a functional cytoskeleton, as shown in vertebrate blood cells (Rosales et al., 1994; Yano et al., 1994). Cytochalasin D-treated haemocytes (Fig. 1C) were morphologically and functionally similar to haemocytes from parasitized caterpillars (Fig. 1B), which suggests that intact actin filaments are required for haemocyte activation. This provided a set of cellular criteria to test whether polydnavirus-mediated haemocyte inactivation is due to the single protein CrV1.

**Recombinant CrV1**

To produce recombinant CrV1, the cDNA fragment coding for the protein (Asgari et al., 1996) was cloned into a baculovirus expression vector and expressed in Sf21 cells (Fig. 3a, lanes 1 and 2). Since the deduced protein sequence predicted a secreted CrV1 protein with potential O- and N-glycosylation sites (Asgari et al., 1996), the cell culture medium was analysed, using lectins, for the presence of recombinant CrV1.
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On Western blots, the GalNAc-specific lectin (HPL) bound to a glycoprotein of 45 kDa which was not present in the culture medium of cells infected with the parental baculovirus (Fig. 3a, lanes 3 and 4). HPL-affinity-purified medium contained a major glycoprotein of the expected size, which was not detected in the control by Western blotting (see Fig. 5), nor by Coomassie staining gel or \(^{35}S\)methionine labelling (not shown). Finally, a similar glycoprotein was identified in haemocytes from parasitized caterpillars (Fig. 3a, lane 6) but was absent in non-parasitized caterpillars (Fig. 3a, lane 5).

To confirm the identity of the CrV1 protein, antibodies were raised against the affinity-purified glycoprotein and tested on polydnavirus-infected haemocytes and cell-free haemolymph (Fig. 3b). At 6 h after parasitization, 45 kDa CrV1 was detected in haemocytes and in cell-free haemolymph from parasitized caterpillars on Western blot (Fig. 3b, lanes 1 and 3), but not in preparations from non-parasitized caterpillars (Fig. 3b, lanes 2 and 4).

**CrV1-mediated haemocyte inactivation**

To test whether the recombinant glycoprotein caused haemocyte inactivation, virus-free culture media from recombinant and parental baculovirus-infected cells were injected into caterpillars, and haemocytes were inspected at various times after injection. Whereas the effects of culture medium from parental baculovirus-infected cells were indistinguishable from those observed in normal caterpillars, the medium from recombinant baculovirus-infected cells caused changes in haemocyte behaviour with regard to spreading and HPL binding which were identical to haemocyte alterations from polydnavirus-infected caterpillars. In this context, the actin filaments of CrV1-treated haemocytes were analysed using phalloidin as a diagnostic marker. In haemocytes from recombinant CrV1-injected caterpillars, the actin filaments were disrupted and unpolymerized actin was detected at the cell periphery and nucleus (Fig. 4B). HPL staining was also absent from infected haemocytes (Fig. 4D).

The absence of HPL binding to haemocytes from CrV1-injected caterpillars (Fig. 4D) is particularly interesting, given that CrV1 is able to bind to HPL (see below). Although these effects were not observed in haemocytes from caterpillars that were injected with culture medium from parental baculovirus-infected cells (Fig. 4A, C), indirect involvement of baculovirus proteins in the observed haemocyte alterations was not entirely excluded in these experiments.

Therefore, to demonstrate that CrV1 alone is sufficient to cause haemocyte inactivation, the CrV1-coding DNA fragment was cloned into an expression plasmid under an immediate early baculovirus promoter, capable of initiating...
transcription in lepidopteran cells (Jarvis et al., 1990). To identify the CrV1 gene product in the culture medium of transfected cells, the glycoprotein was enriched using an HPL column and bound glycoproteins were eluted from the column with a GalNAc sugar solution (Fig. 5a; HPL), and their identity was confirmed with anti-CrV1 antiserum (Fig. 5a; AB). Injection of the plasmid-derived recombinant CrV1 glycoprotein into caterpillars caused haemocyte changes that were indistinguishable from those observed with the baculovirus-derived protein (Fig. 6B, F), as well as polydnavirus-infected haemocytes (Asgari et al., 1996). The inactivation of haemocytes can be detected in caterpillars for up to 30 h after CrV1 injection. Haemocytes isolated after this time are normal in most caterpillars. Similarly, haemocytes isolated from caterpillars 2 days after parasitization are indistinguishable from normal haemocytes. This suggests that the transient inactivation of haemocytes by polydnavirus infection of caterpillars is mediated by a single extracellular glycoprotein.

Given that CrV1 is expected to interact with the cell surface, the absence of HPL binding to CrV1-treated haemocytes was unexpected. Using antibodies against the CrV1 and HPL, recombinant CrV1 was analysed in the presence and absence of haemolymph (Fig. 5b; HPL). CrV1 treated with cell-free haemolymph did not bind HPL. This is probably not due to protein degradation, since antibody staining of haemolymph-treated CrV1 showed only slight effects on the size and amount of the protein (Fig. 5b; AB). This suggests that CrV1 is not degraded but is modified by cell-free haemolymph components producing a small but significant modification which alters the ability to bind HPL. Although the functional implications of this observation are not clear, it may explain the lack of HPL binding from CrV1-injected caterpillars.

**Discussion**

This study shows that the polydnavirus-mediated effects on haemocytes observed in parasitized caterpillars are probably caused by a single glycoprotein, CrV1. Polydnavirus-infected caterpillars and caterpillars injected with recombinant CrV1 revealed similar haemocyte alterations which are consistent with inhibition of immune-related cellular activation processes. In parasitized caterpillars, the CrV1 glycoprotein is secreted into the haemolymph by polydnavirus-infected haemocytes and fat body cells. Moreover, recombinant CrV1 shows its
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KDa 50 36
30 16 6

AB

Rec+H Rec

MW

Fig. 5. (a) Plasmid-encoded CrV1. Culture medium from Sf21 cells was passed through an HPL–Sephadex column and the bound fraction eluted with GalNAc solution. Aliquots of the eluted material were analysed on Western blots and probed with peroxidase-conjugated HPL (left) and anti-CrV1 antiserum (AB; right). Lane 1, culture medium from vector plasmid transfected cells (15 µl eluate, equivalent of 0.7 ml medium); lane 2, culture medium from recombinant plasmid-transfected cells (15 µl eluate, equivalent of 0.7 ml medium); lane 3, culture medium from recombinant baculovirus-infected cells (15 µl concentrate, equivalent of 150 µl medium). (b) Haemolymph-treated CrV1. Aliquots of culture medium containing the recombinant protein (Rec) were incubated with cell-free haemolymph of P. rapae (Rec + H) and analysed on a Western blot probed with peroxidase-conjugated HPL (left) and anti-CrV1 antiserum (AB; right). The protein is not recognized by the lectin after treatment with haemolymph.

effects on haemocytes only in conjunction with haemolymph, but not when applied to haemolymph-free haemolymph, implying extracellular modifications to the protein. Removal of HPL-binding sugars from the glycoprotein by haemolymph components could suggest a sugar-dependent uptake of the glycoprotein by haemocytes which is dependent on removal of a terminal GalNAc. N-Acetylgalactosamidase activity has indeed been reported in insects (Vandie et al., 1996). It is possible that secretion and extracellular modification are prerequisites for CrV1 to interact with a specific receptor on the non-activated haemocyte surface. Alternatively the modified glycoprotein may be internalized to interact directly with the actin filament in the cytoplasm. The exact mode of CrV1 interaction with the haemocyte remains to be elucidated.

Although nothing is known about the molecular reactions leading to haemocyte activation, these events are essential for the attachment, aggregation (Gupta, 1991; Ratcliffe, 1993) and coagulation reactions (Bohn, 1986) that constitute the cellular defence response against bacteria and parasitoids. Exposure of haemocytes to foreign objects or microorganisms causes cell membrane rearrangements (Gupta, 1991; Nappi & Silvers, 1984; Strand & Pech, 1995 a), which expose specific surface molecules and facilitate cell adhesion (Rizki & Rizki, 1983). These changes may also include the discharge of cortical granules containing components resembling extracellular matrix components (Strand & Pech, 1995 a).

The discharge and exposure of adhesive components probably enables haemocytes to attach to foreign surfaces. In addition, the formation of microparticles has recently been shown to be part of insect haemocyte activation (Theopold & Schmidt, 1997). Activated haemocytes form blebs and cellular extensions which lead to vesicle formation, and which are similar to microparticles in activated mammalian blood cells (Yano et al., 1994). A characteristic feature of particle formation is the exposure of PS on the outer leaflet of the cellular and particle membrane, which can be identified using annexin V as a diagnostic marker. The presence of PS is known to enhance coagulation processes in vertebrate systems. Since both degranulation and microparticle formation involve a rearrangement of the cytoskeleton (Rosales et al., 1994; Yano et al., 1994), the absence of functional actin filaments in CrV1-treated haemocytes is probably the primary cause of haemocyte inactivation. More specifically, the inactivation of actin filaments by CrV1 probably precludes cellular activation reactions of haemocytes similar to cytochalasin D inactivation in vertebrate blood cells (Bengtsson et al., 1993; Mooney et al., 1995).

The effects of CrV1 on haemocytes are reversible. After 2–3 days the haemocytes have virtually returned to normal activation behaviour, both in parasitized caterpillars and in caterpillars injected with recombinant CrV1 protein. In contrast to other systems, no evidence for cell death (Rizki & Rizki, 1990) or apoptosis (Strand & Pech, 1995 b) of haemocytes is observed in P. rapae caterpillars. Instead haemocytes recover
from the polydnavirus- and CrV1-mediated effects, as judged by Hoechst staining (not shown) and annexin V labelling. Moreover, in Hoechst-stained haemocyte preparations increased mitotic activity was not detected following parasitization or CrV1 injection, arguing against a possible replacement of inactivated haemocytes by an increased proliferation of a non-affected cell population. This, and direct observations of haemocytes at various time periods after CrV1-application, indicate that haemocytes gradually recover from CrV1-mediated alterations, suggesting that the CrV1-induced haemocyte changes are reversible. CrV1-mediated inactivation of haemocytes is transient and probably affects specific immune functions related to cell-mediated defence reactions; the protein thus has the potential to become a useful tool in basic and applied research. The mechanism of how the wasp larva is protected after the host has regained its cellular immune defence is not known and remains to be investigated.

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