Synthesis and toxicity of full-length and truncated bacterial CryIVD mosquitocidal proteins expressed in lepidopteran cells using a baculovirus vector

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Full-length (72K) and truncated (61K) CryIVD mosquitocidal proteins of Bacillus thuringiensis (Bt) were expressed in Spodoptera frugiperda cells and larvae of Trichoplusia ni using a baculovirus vector to investigate the role of CryIVD peptides in toxicity as well as to evaluate further the baculovirus/lepidopteran system for expressing Bt proteins. The cryIVD genes were inserted into the Autographa californica multinucleocapsid nuclear polyhedrosis virus (AcMNPV) under control of the polyhedrin promoter by recombination in S. frugiperda cells between a transfer vector carrying the Bt genes and vDA26Z, a recombinant AcMNPV carrying the Escherichia coli β-galactosidase gene under control of the DA26 promoter. Recombinant AcMNPVs carrying the genes were detected as blue occlusion body-negative plaques in monolayers of S. frugiperda cells grown in the presence of X-Gal. Infection of S. frugiperda cells and T. ni larvae with plaque-purified recombinant virus, expressing either the full-length or truncated CryIVD protein, resulted in the synthesis of proteins of the expected size, as confirmed by immunoblot analyses, and their crystallization into cuboidal inclusions in the cytoplasm. Infected cells and purified inclusions from the virus (AcCryIVD) expressing the full-length protein were highly toxic to mosquito larvae, but similar preparations from the virus (AcCryIVD-C) expressing the truncated protein with a 9-6K deletion at the N terminus were non-toxic. Proteolysis with trypsin of CryIVD proteins produced by Bt and the recombinant AcMNPVs yielded peptides corresponding in size, showing that synthesis of mosquitocidal Bt proteins in lepidopteran cells occurred. The lack of toxicity of the truncated CryIVD protein, which like the toxic full-length protein yielded a 34K protein on proteolysis that has been implicated in toxicity, indicates that by itself this protein is non-toxic. These results demonstrate the utility of the baculovirus system for expression of mosquitocidal Bt proteins and for investigation of their mode of action.

Introduction

Pressure to eliminate organic chemical insecticides from insect control programmes continues to mount, due primarily to the development of resistance in many insect pests and concern that continued use of these chemicals will increasingly contaminate the environment, particularly the food and water supply. Many alternatives to chemical insecticides are under consideration and among these are pathogens that cause fatal diseases specific to insects (Payne, 1988). The most promising pathogens include the bacterium Bacillus thuringiensis (Bt) and insect baculoviruses, especially the nuclear polyhedrosis viruses (NPVs). Bt consists of a complex of subspecies, many of which produce proteins that are insecticidal (Hofte & Whiteley, 1989), whereas the NPVs are large double-stranded DNA viruses that cause fatal diseases of insects, especially of lepidopterous insects (Matthews, 1982; Granados & Federici, 1986).

Several pathogens belonging to these two groups are already produced commercially and used as microbial insecticides (Burges, 1981; Payne, 1988). Advantages favouring further use and development include their relative ease of mass production, good efficacy in many cases, safety for non-target organisms, and that they can be applied to crops using conventional application technologies. There are, however, several disadvantages. Many insect pests for which there are no cost-effective Bts or NPVs are known. Moreover, even for pests for which NPVs are available, the viruses often take a week or longer to kill larvae advanced in development, by which time economic damage to crops can be substantial (Huber, 1986; Payne, 1988).
Potential solutions to these problems include the discovery or development of new strains of Bt with novel or broader host ranges and the discovery or development of viruses that kill insects soon after infection. With respect to the latter, the discovery of the Autographa californica multinucleocapsid NPV (AcMNPV) provided a virus with a relatively broad host range among lepidopterous insects, yet this virus remains inadequate as an insecticide because, like other NPVs, it often takes too long to kill insects at rates sufficient to be economic under field conditions.

Over the past decade there have been considerable advances in our knowledge of the molecular biology of both Bts and NPVs (Hofte & Whiteley, 1989; Luckow & Summers, 1988; Miller, 1988; Possee & Howard, 1987). This knowledge provides a base for developing technologies for engineering more effective viral and bacterial insecticides. For NPVs, important requirements for engineering more effective viruses are the identification of proteins that will kill the insect quickly, or at least cause a rapid paralysis that prevents or inhibits feeding, and the development of systems for the construction and efficient screening of recombinant viruses that express these proteins. Insecticidal proteins produced by Bt are candidates for use in baculovirus insecticides because, in their activated form, they are known to be toxic upon injection (Heimpel, 1967). In two recent studies, CrylA (133K) proteins specific for lepidopterous insects were expressed in vitro in cells of the fall army worm Spodoptera frugiperda (Sf) using baculovirus vectors, and the resulting Bt endotoxin-containing cells were shown to be toxic to caterpillars upon ingestion (Martens et al., 1990; Merryweather et al., 1990). In the present study, we engineered the AcMNPV to express either the full-length crylVD gene, which encodes a 72K mosquitocidal protein, or a truncated version of this gene encoding a 61K peptide. The genes were expressed under control of the polyhedrin promoter in Sf cells and in larvae of the cabbage looper Trichoplusia ni. We chose this protein and expression system to investigate the toxicological properties of the full-length and truncated proteins as well as to evaluate further the utility of the baculovirus/lepidopteran system for expressing Bt proteins.

**Methods**

**Bacterial strains and vectors.** All gene constructs were made using the Escherichia coli vector pGEM-1 (Promega) in general following procedures recommended in Sambrook et al (1989). For cloning products prepared using the polymerase chain reaction (PCR) and for DNA sequencing, the phagemid pBLUESCRIPT-KS (Stratagene) was used. The E. coli strain JM105 was used to grow recombinant pGEM-1 plasmids, and E. coli strain XL1-Blue to grow recombinant pBLUESCRIPT-KS phagemids. The bacteria were transformed according to the method of Chung & Miller (1988). Recombinant colonies were selected on LB agar plates containing 50 μg/ml of ampicillin; minipreparations were made as described by Holmes & Quigley (1981).

**Enzymes and reagents.** Restriction enzymes, T4 DNA ligase, calf intestine alkaline phosphatase, X-Gal and the Random Primed DNA Labelling Kit were from Boehringer Mannheim. Sequencing was performed with Sequenase (United States Biochemical) by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a double-stranded template in pBLUESCRIPT-KS. The Taq polymerase as well as the PCR kit were from Perkin Elmer-Cetus.

**Construction of pAcP1 and pAcP2.** Plasmids used to develop transfer vectors for inserting genes into the AcMNPV under control of the polyhedrin promoter. The polyhedrin gene and its promoter were obtained on a 3.1 kb BamHI fragment isolated from the transfer vector pGB-B6874/Sal (Pennock et al., 1984) as illustrated in Fig. 1. This fragment contained the AcMNPV F, V and T HindIII fragments reconstructed so that the promoter and the first 171 base pairs of the polyhedrin gene (57 amino acids) were located on one end of the 3.1 kb BamHI fragment, with the rest of the gene on the other end in the opposite orientation. This fragment was cloned into the BamHI site of pGEM-1 in the two possible orientations to yield the plasmids pAcP1 and pAcP2. In pAcP1, the polyhedrin sequence was located upstream from the cloning sites Smal, XmaI, AvaI, EcoRI and SacI, whereas in pAcP2 the available cloning sites were XbaI, AccI and PstI.

**Fig. 1.** Construction of pAcP1, a generalized transfer vector for inserting genes into AcMNPV under control of the polyhedrin promoter. The BamHI fragment from pGP-B6874/Sal containing the F, V and T HindIII fragments of AcMNPV flanked by sections of the polyhedrin gene was ligated into the BamHI site of pGEM-1. Origins of replication (Ori), ampicillin resistance genes (Amp') and kanamycin resistance genes (km') are indicated.
Origins of replication and ampicillin resistance genes are indicated as gene of Bt subsp., Fig. 2. Construction of pCrylVD1, a plasmid containing the crylVD cloned into the plasmid pAcP1 to yield the recombinant plasmid pCrylVD1 (Fig. 2). After filling in of the ends with Klenow enzyme, this fragment was cloned into the Smal site of pGEM-1 after filling in the Clal end by Klenow repair to yield the recombinant plasmid pCrylVD1 (Fig. 2).

Construction of ptAcPCrylVD-C, a transfer vector containing the crylVD gene with a deletion at the 5' end. To produce a truncated CrylVD protein which on proteolytic cleavage in vivo or in vitro would yield the abundant 34K peptide, a fragment of the crylVD gene encoding this peptide was cloned into the plasmid pAcPl to yield the transfer vector ptAcPCrylVD-C. For this, a 3.5 kb BamHI–SacI fragment of pCrylVD1 encoding the entire crylVD gene was purified by elution from an agarose gel and then digested with TaqI and then specifically amplified the 400 bp fragment by PCR. The second primer contained the SacI site naturally occurring in crylVD (underlined). For the reaction 1 ng of template DNA was amplified during 25 cycles in a Perkin Elmer Cetus DNA thermocycler using the following conditions: denaturation, 94 °C for 1 min; annealing, 37 °C for 2 min; extension, 72 °C for 3 min. The extension was performed for 15 min at 72 °C after completion of the last cycle. The PCR product was purified in an agarose gel, and then cloned and sequenced using pBLUESCRIPT-KS to check the accuracy of the synthesis.

Construction of ptAcPCrylVD-I, a transfer vector with the Bt crylVD gene under control of the polyhedrin promoter. After modification and reconstruction of the crylVD gene, the Smal–BglI fragment of pCrylVD3, which contained the coding region of crylVD, was cloned into the Smal–BglI site of pAcPl yielding the transfer vector ptAcPCrylVD (Fig. 3b).

Insect cells and viruses. The cell line IPLB-SF-21 (Vaughn et al., 1977) established from Sf, was used as the expression host. The cells were maintained at 28 °C in Grace’s medium ( Gibco) supplemented with 10% foetal bovine serum, 0.16% TC-yeastolate, 0.2% lactalbumin hydrolysate and 0.16% tryptose broth (Difco). The engineered baculovirus vDA26Z (O’Reilly et al., 1990) carrying the E. coli lacZ gene under the control of the DA26 promoter was used in place of a wild-type (AcMNPV) during the cotransfection experiments. Another recombinant, AcMNPV-LIGP-gal3 (Pennock et al., 1984), which is polyhedrin-negative and carries a polyhedrin–lacZ fusion gene, was used as a control for some experiments. Wild-type and recombinant viruses were produced in cells grown as monolayers in either 25 or 75 cm² Falcon flasks.

Plaque assays. Plaque assays were carried out in 60 × 15 mm Petri dishes essentially as described by Lee & Miller (1978). The infected cells were overlaid with the supplemented Grace’s medium described above to which was added 0.5% SeaPlaque agarose (Seakem), 160 µg/ml of X-Gal and 25 µg/ml of each antibiotic, ampicillin and kanamycin. Plaque forming units were detected as blue spots or by staining with 0.05 % (w/v) neutral red (Brown & Faulkner, 1977) 4 days post-infection (p.i.) at 28 °C.

DNA transfection and selection of recombinant virus. The Sf cells were transfected with a 10:1 mixture of the transfer vector (ptAcPCrylVD-C or ptAcPCrylVD-I) DNA and vDA26Z DNA by calcium phosphate precipitation (Potter & Miller, 1980). After incubation for 4 days at 28 °C, the supernatant was harvested and the concentration of p.f.u. was determined by titration in semi-confluent layers of Sf cells as described above. Polyhedrin-negative blue plaques were visualized using an inverted light microscope. Representative plaques were collected, placed individually in 1 ml of Grace’s medium, and purified through three cycles of plaque purification. The recombinant viruses from each plaque were then amplified in Sf cells, first in 24-well plates and then in 25 cm² tissue culture flasks to yield virus stocks with titres of 10⁷ to 10⁸ p.f.u./ml. The recombinant viruses were named vAcCrylVD-C (expressing the 5'-deleted CrylVD gene) and vAc-CrylVD-I (expressing the full-length gene).

Purification of Bt inclusions from infected Sf cells and insects. The crystalline inclusions of Bt protein resulting from expression of the crylVD gene in insect cells and larvae were purified by differential centrifugation and on sodium bromide gradients, in the latter case where greater purity was required. To isolate inclusions from Sf cells, at

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**Fig. 2.** Construction of pCrylVD1, a plasmid containing the crylVD gene of Bt subsp. morrisoni. A 3.5 kb Clal–EcoRV fragment containing the full-length crylVD gene was cloned into the Smal site of pGEM-1. Origins of replication and ampicillin resistance genes are indicated as gene of Bt subsp., Fig. 2. Construction of pCrylVD1, a plasmid containing the crylVD gene of Bt subsp. morrisoni (PG-14) encoding the mosquitocidal protein CrylVD (72K) (Frutos et al., 1991) was isolated from the plasmid pMl (Galjart et al., 1987) on a 3.5 kb Clal–EcoRV fragment. This fragment was cloned into the Smal site of pGEM-1 after filling in the Clal end by Klenow repair to yield the recombinant plasmid pCrylVD1 (Fig. 2).

Construction of ptAcPCrylVD-C, a transfer vector containing the crylVD gene with a deletion at the 5' end. To produce a truncated CrylVD protein which on proteolytic cleavage in vivo or in vitro would yield the abundant 34K peptide, a fragment of the crylVD gene encoding this peptide was cloned into the plasmid pAcPl to yield the transfer vector ptAcPCrylVD-C. For this, a 3.5 kb BamHI–SacI fragment of pCrylVD1 encoding the entire crylVD gene was purified by elution from an agarose gel and then digested with TaqI to generate a 21 kb fragment (Fig. 3a). This fragment carried the portion of the crylVD gene from base 267 to 468 bp downstream from the stop codon. After filling in of the ends with Klenow enzyme, this TaqI fragment was inserted into the Avel site of pAcPl, also repaired with Klenow enzyme, yielding ptAcPCrylVD-C. To ensure that the cloning in this transfer vector was in frame, the region of the polyhedrin–crylVD gene fusion was sequenced, and the synthesis of the protein was checked by transcription and translation in vitro.

Transcription and translation in vitro. The transfer vector ptAcPCrylVD-C was linearized by digestion with SacI, and then transcribed in vitro using the Riboprobe Gemini System II (Promega). The resulting mRNAs were translated in vitro using a rabbit reticulocyte, methionine-minus amino acid mixture (Promega) and [35S]methionine (Amersham). Proteins were separated in an 11% SDS-polyacrylamide gel, which was then dried and exposed on X-ray film (Kodak X-Omat) for 3 days at room temperature.

Modification and reconstruction of the full-length crylVD gene using PCR. There were no restriction sites near the S' end of the crylVD gene suitable for cloning the gene into pAcPl or pAcP2 under control of the polyhedrin promoter. Therefore, the gene was modified and reconstructed using PCR to create three new restriction sites, StyI (CCAAAGG), BamHI (GGATCC) and Smal (CCCGGG), in a 400 bp section near the S' end of the gene (Fig. 3b). This was accomplished by synthesizing two primers, 5' CCAAGGATCCGGGGAATGATATTATGGAAGATA 3' and 5' AACCCTTGCGTTTGACAGGG 3', and then specifically amplifying the modified 400 bp fragment by PCR. The second primer contained the StyI site naturally occurring in crylVD (underlined). For the reaction 1 ng of template DNA was amplified during 25 cycles in a Perkin Elmer Cetus DNA thermocycler using the following conditions: denaturation, 94 °C for 1 min; annealing, 37 °C for 2 min; extension, 72 °C for 3 min. The extension was performed for 15 min at 72 °C after completion of the last cycle. The PCR product was purified in an agarose gel, and then cloned and sequenced using pBLUESCRIPT-KS to check the accuracy of the synthesis.

Construction of ptAcPCrylVD-I, a transfer vector with the Bt crylVD gene under control of the polyhedrin promoter. After modification and reconstruction of the crylVD gene, the Smal–BglI fragment of pCrylVD3, which contained the coding region of crylVD, was cloned into the Smal–BglI site of pAcPl yielding the transfer vector ptAcPCrylVD (Fig. 3b).

Plaque assays. Plaque assays were carried out in 60 × 15 mm Petri dishes essentially as described by Lee & Miller (1978). The infected cells were overlaid with the supplemented Grace’s medium described above to which was added 0.5% SeaPlaque agarose (Seakem), 160 µg/ml of X-Gal and 25 µg/ml of each antibiotic, ampicillin and kanamycin. Plaque forming units were detected as blue spots or by staining with 0.05 % (w/v) neutral red (Brown & Faulkner, 1977) 4 days post-infection (p.i.) at 28 °C.

DNA transfection and selection of recombinant virus. The Sf cells were transfected with a 10:1 mixture of the transfer vector (ptAcPCrylVD-C or ptAcPCrylVD-I) DNA and vDA26Z DNA by calcium phosphate precipitation (Potter & Miller, 1980). After incubation for 4 days at 28 °C, the supernatant was harvested and the concentration of p.f.u. was determined by titration in semi-confluent layers of Sf cells as described above. Polyhedrin-negative blue plaques were visualized using an inverted light microscope. Representative plaques were collected, placed individually in 1 ml of Grace’s medium, and purified through three cycles of plaque purification. The recombinant viruses from each plaque were then amplified in Sf cells, first in 24-well plates and then in 25 cm² tissue culture flasks to yield virus stocks with titres of 10⁷ to 10⁸ p.f.u./ml. The recombinant viruses were named vAcCrylVD-C (expressing the 5'-deleted CrylVD gene) and vAc-CrylVD-I (expressing the full-length gene).

Purification of Bt inclusions from infected Sf cells and insects. The crystalline inclusions of Bt protein resulting from expression of the crylVD gene in insect cells and larvae were purified by differential centrifugation and on sodium bromide gradients, in the latter case where greater purity was required. To isolate inclusions from Sf cells, at
3 days p.i. approximately 1 × 10^6 cells were incubated for 2 min at room temperature in 100 μl of lysis buffer (50 mM-Tris-Cl pH 8, 20 mM-EDTA, 2.5% SDS, 5% Triton X-100 and 30% glycerol). After dilution in 1 ml of 30% glycerol, the disrupted cells were centrifuged for 2 min in a microfuge and the pellet, which contained the crystals, was washed twice with PBS pH 7-5.

To obtain the larger amounts of CrylVD inclusions needed for bioassays, 1 ml of infected cells at 3 days p.i. was treated with 2 ml lysis buffer for 2 min, diluted to 40 ml with 30% glycerol, and then centrifuged at 25000 g for 20 min. The pellet was washed with PBS and resuspended in 1-5 ml of 1% Triton X-100. This suspension was then layered on a 26 to 36% sodium bromide gradient, and the gradients were centrifuged in an SW41 rotor at 28000 r.p.m. for 90 min at 4°C. The crystals, which formed a distinct band in the gradient, were removed from the tube with a Pasteur pipette, diluted in PBS, and pelleted by centrifugation.

To obtain CrylVD inclusions from infected T. ni, the larvae were collected after death and triturated in PBS in a glass tissue grinder. The resultant slurry was filtered through a 100-mesh nylon screen, and the particulates were sedimented by centrifugation at 1700 g for 10 min at room temperature. The pellet was resuspended in two volumes of lysis buffer and the inclusions were purified on NaBr gradients as described above.

**Proteolysis with trypsin.** Purified inclusions from both infected insect cells and larval tissues as well as from expression of the crylVD gene in Bt subsp. kurstaki (C. Chang, S. M. Dai, R. Frutos, B. Federici & S. S. Gill, unpublished results) were treated with 50 mM-Na,CO₃ pH 11.0 for 1 h at 37°C. The suspensions were neutralized with 2.0 M-HCl and incubated for 2 h at 37°C with 40 μg/ml of trypsin (Sigma) (Chilcott & Ellar, 1988; Gill et al., 1987; Ibarra & Federici, 1987).

**Analysis of protein by SDS–PAGE and immunoblotting.** SF cells or larval tissues of T. ni infected with the recombinant viruses as well as Bt inclusions purified from these were lysed in electrophoresis sample buffer (100 μl Tris–HCl pH 6-8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.01% bromophenol blue), incubated for 5 min in boiling water, and separated in 11% polyacrylamide gels at 60 V overnight (Laemmli, 1970). Gels were stained with 0.1% Coomassie Blue R-250 in 7% (v/v) acetic acid containing 50% methanol. For immunodetection, proteins were transferred in a semi-dry blotter (Bio-Rad) onto a Immobilon-P membrane (Millipore) and probed with an antibody directed against the CrylVD protein of Bt subsp. israelensis. The detection was performed using a ProtoBlot AP System (Promega).

**DNA labelling and hybridization.** A Smal–SacI fragment carrying the entire coding region of the modified crylVD gene was isolated by electrophoresion and labelled with [32P]dCTP using random oligonucleotides as primers (Random Primed DNA Labelling Kit, Boehringer Mannheim). After digestion by restriction endonucleases and separation by agarose gel electrophoresis, viral and plasmid DNAs were transferred onto a nylon membrane (Southern, 1975) and incubated overnight at 65°C with the probe. The membrane was then exposed on the X-ray film (Kodak) at room temperature for 20 min.

**Light and electron microscopy.** Infected or healthy cells were observed and photographed using a compound upright or inverted phase light microscope (Zeiss). For electron microscopy, healthy and infected cells, and purified inclusions, were embedded in 2% agar, and then fixed in 3% glutaraldehyde in 0.1 M-cacodylate buffer (2 h) followed by post-fixation in 1% OsO₄ in the same buffer. The specimens were dehydrated through an ethanol–propylene oxide series and embedded in Epon–Araldite. Ultrathin sections were cut on a Sorval MT 5000 ultramicrotome, stained with lead citrate and uranyl acetate, and examined and photographed with a Hitachi 600 electron microscope.

**Insects and bioassays.** The toxicity of the CrylVD proteins expressed by the baculovirus vectors was determined through bioassays of infected SF cells, larval tissues and purified crystals from these against first instars of the mosquito Aedes aegypti. In addition, the recombinant virus expressing the CrylVD protein was assayed against larvae of T. ni by injecting or feeding them virus. For the assays against mosquitoes, SF cells infected at a ratio of 2 p.f.u. per cell were collected at 72 p.i. For vAcCrylVD tissue from T. ni, the fat body was collected 3 days p.i. The samples were washed twice with PBS, sonicated (four times, 10 s each), and the protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce). Then the samples were diluted over a range of concentrations and assayed against first instars of A. aegypti (Ibarra & Federici, 1987). Bioassays were repeated three times, using three different lots of mosquito larvae, with 50 larvae per replicate. Mortalities were scored 24 h after treatment and the 50% lethal concentration values (LC₅₀) were determined by probit analysis (Finney, 1962). Controls consisted of fat body tissue from uninfected larvae and SF cells and larvae and cells infected with the AcMNPV-vD2A62.

Bioassays on T. ni larvae were performed by injecting early fourth instar larvae with a suspension containing non-occluded virions or per os using polyhedra purified from SF cells coinfected with vAcCrylVD and vD2A6Z at a ratio of 1:1 (in which both viral nucleocapsids are occluded in polyhedra; Kuroda et al., 1989).

**Results**

**Construct rationale**

The 72K CrylVD protein differs from all other Bt proteins in that, when it is cleaved by proteases, it yields several peptides with M/ values ranging from 30K to 40K (Ibarra & Federici, 1986; Chilcott & Ellar, 1988). When fed to mosquito larvae, or assayed in vitro against cells of the lepidopteran, Mamestra brassicae, or cells from a variety of mosquito species, this mixture of peptides causes mosquito death or cell lysis at concentration of around 100 μg/ml (Chilcott & Ellar, 1988). The two most abundant peptides resulting from cleavage of the 72K

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Fig. 3. Construction of the transfer vectors (a) ptAcPCrylVD-C, (b) ptAcPCrylVD, and (c) illustration of chimeric polyhedrin–CrylVD proteins they expressed. (a) A 2.7 kb TaqI fragment obtained from pCrylVD1 was cloned into pAcP1. (b) A 400 bp fragment of the crylVD gene was modified to create three new restriction sites using PCR and the plasmid pCrylVD1 as a template. The fragment was then cloned into the SglI site of pCrylVD1 yielding pCrylVD2 which then contained both the original and modified 400 bp fragments. Digestion of pCrylVD2 with BamHI followed by ligation yielded pCrylVD3 in which the original 400 bp fragment was eliminated. The 3-7 kb fragment containing the complete crylVD gene and the deleted portions of the ampicillin resistance gene (Amp') and origin of replication (ori) was then isolated from pCrylVD3 by digestion with the same enzymes and ligated into pAcP1. This yielded ptAcCrylVD1, containing a chimeric polyhedrin–crylVD gene under control of the polyhedrin promoter, and in which the origin of replication and ampicillin resistance gene were restored. (c) Schematic illustration of polyhedrin–CrylVD and polyhedrin–CrylVD-C proteins indicating the relative position of the protease cleavage site and the size of the fragment resulting from proteolysis of either protein.
protein are those of 38K and 34K, with the latter being the most abundant, suggesting that it might be the activated toxin. Peptide sequencing of the N terminus of the 34K peptide revealed that this protein was cleaved from the C terminus of CrylVD when the latter was digested with trypsin. More specifically, the N terminus of the 34K cleavage product had the amino acid sequence Tyr-Gln-Asn-Pro-Asn-Asn-Glu (S. S. Gill & S. M. Dai, personal communication) which corresponded with the amino acid sequence of the CrylVD protein beginning at amino acid 349, with the deduced size (33.2K) and location being obtained from the gene sequence (Frutos et al., 1991). This implied that the 34K fragment might be produced and tested for toxicity by truncating the 72K protein at the N terminus and feeding it to mosquito larvae. A moderately truncated protein, provided it folded properly, could be expected to yield a 34K peptide upon proteolytic cleavage in vivo that would be the same as the cleavage product of the same size from the native protein. To test this, our first strategy was to construct such a protein by taking advantage of a TaqI site near the 5' end of the crylVD gene. Cleaving the gene at this site enabled us to eliminate a portion of the gene that encoded a 9-7K fragment at the N terminus of the CrylVD protein, and fuse the truncated gene in frame with a portion of the polyhedrin gene including the polyhedrin promoter. Significantly, this deleted gene fragment encoded in part a highly hydrophobic domain. In addition, and for comparative purposes, we developed an alternative strategy for expressing the full-length 72K CrylVD protein. These strategies and the principal steps used to construct the transfer vectors and recombinant viruses are illustrated schematically in Fig. 1 to 3.

Construction of the plasmids pAcP1, pAcP2, the transfer vector ptAcCrylVD-C and the recombinant virus vAcCrylVD-C

Screening recombinant plasmids with restriction endonucleases identified the plasmids pAcP1 and pAcP2, as well as the transfer vector, ptAcPCrylVD-C (Fig. 1 to 3a). Cotransfection of SF cells with the transfer vector ptAcCrylVD-C and AcMNPV (vDA26Z) resulted in the recombinant virus vAcCrylVD-C, which expressed the truncated crylVD gene of Bt in both SF cells and larvae of T. ni (Fig. 4). The recombinant virus was detected as blue plaques in which the cell nuclei contained no polyhedra. After isolation, the plaques were purified three times, and the recombinant viral DNA was analysed by restriction endonuclease analysis and by hybridization using the crylVD gene as a probe; this confirmed the insertion of the gene into the recombinant virus.

When expressed in either SF cells or larvae of T. ni, the truncated CrylVD protein formed crystalline cuboidal inclusions in the cytoplasm of cells, but neither the infected cells nor purified crystals were toxic to mosquito larvae, despite the formation of the expected 33K protein upon proteolytic cleavage (Fig. 4; Table 1).

Table 1. Toxicity of cells and purified inclusions from cells infected with Autographa california MNPV expressing a truncated or full-length crylVD gene of Bt

<table>
<thead>
<tr>
<th>Sample</th>
<th>LC$_{30}$ (ng protein/ml)*</th>
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<tbody>
<tr>
<td>vAcCrylVD-C</td>
<td></td>
</tr>
<tr>
<td>Inclusions from SF cells</td>
<td>&gt;4000†</td>
</tr>
<tr>
<td>Infected SF cells</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>SF cell controls‡</td>
<td>Non-toxic†</td>
</tr>
<tr>
<td>Inclusions from T. ni</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>Infected T. ni cells</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>T. ni cell controls‡</td>
<td>Non-toxic†</td>
</tr>
<tr>
<td>vAcCrylVD</td>
<td></td>
</tr>
<tr>
<td>Inclusions from SF cells</td>
<td>224 ± 42</td>
</tr>
<tr>
<td>Infected SF cells</td>
<td>1892 ± 799</td>
</tr>
<tr>
<td>SF cell controls‡</td>
<td>Non-toxic†</td>
</tr>
<tr>
<td>Inclusions from T. ni</td>
<td>&gt;253 ± 53</td>
</tr>
<tr>
<td>Infected T. ni cells</td>
<td>3251 ± 678</td>
</tr>
<tr>
<td>T. ni cell controls‡</td>
<td>Non-toxic†</td>
</tr>
</tbody>
</table>

* Against first instars of A. aegypti; three replicates, 50 larvae/replicate.
† No toxicity detected at 4 µg/ml, the highest concentration tested.
‡ Includes cells infected with AcMNPV vDA26Z and LIGP-gal3 (polyhedrin-minus).
In vitro modification of the crylVD gene using PCR, and construction of the transfer vector ptAcCrylVD and the recombinant virus vAcCrylVD

To determine whether the full-length CrylVD protein produced in Sf cells or larvae would be toxic, we then inserted the full-length gene into vDA26Z. Because there were no convenient cloning sites near the 5' end of the gene, we modified the gene as described below to make the transfer vector ptAcPCrylVD.

The crylVD gene was modified in vitro using synthetic primers and PCR to create three new restriction sites for StyI, BamHI and SmaI (Fig. 3b). The plasmid pCrylVD1 containing the crylVD gene was used as a template for the synthesis. Amplification yielded a 400 bp fragment with a naturally occurring StyI site on the 3' end, and the StyI, BamHI and SmaI sites on the 5' end located almost immediately upstream from the ATG. The latter sites were created using the PCR primer. The PCR product was cloned and sequenced to ensure the accuracy of the synthesis, and then cleaved with StyI and inserted into pCrylVD1 digested with the same enzyme.

The resulting plasmid, pCrylVD2, was checked to ensure the insert was oriented correctly, and to confirm that it carried a modified crylVD gene with both the original and modified 400 bp 5' region. Digestion of this plasmid with BamHI followed by ligation yielded a new plasmid, pCrylVD3, in which the crylVD promoter, the original 400 bp 5' region and the de novo synthesized StyI site were eliminated. This plasmid then contained a crylVD gene identical to the native gene except that it had an engineered SmaI site upstream from the ATG, enabling in-frame cloning of the crylVD gene with the truncated polyhedrin gene of the plasmid pAcP1 (Fig. 1 and 3b). To do this, pAcP1 was cleaved with SmaI and BglI which resulted in loss of the origin of replication from pGEM-1 and a portion of the ampicillin resistance gene. The 3-7 kb fragment (containing the complete crylVD gene and the deleted portions of the ampicillin resistance gene and origin of replication) was then isolated from pCrylVD3 by digestion with the same enzymes and ligated into pAcP1. This yielded the transfer vector ptAcCrylVD (Fig. 3b), containing a
chimeric polyhedrin–cryIVD gene under control of the polyhedrin promoter, and in which the origin of replication and ampicillin resistance gene were restored.

Screening recombinant plasmids with restriction endonucleases identified the transfer vector ptAcPCryIVD which contained the full-length gene in the proper orientation. This result was confirmed by restriction enzyme analysis and hybridization using the cryIVD gene as a probe (Fig. 5). Cotransfection of Sf cells with ptAcPCryIVD and AcMNPV (vDA26Z) resulted in the recombinant virus vAcCryIVD which expressed the full-length cryIVD gene of Bt in both Sf cells and larvae of T. ni. As with vAcCryIVD-C, the recombinant virus was detected as blue plaques in which the cell nuclei contained no polyhedra. The plaques were purified as above, and the recombinant viral DNA was analysed by restriction endonuclease analysis as well as by hybridization using the cryIVD gene as a probe to confirm insertion of the gene into the recombinant virus (Fig. 5).

In contrast to the results obtained with the truncated gene, expression of the full-length cryIVD gene resulted in inclusions with significant toxicity to mosquitoes. The expression and properties of this protein were therefore studied in greater detail.

Expression of the CryIVD protein in insect cells

Infection of Sf cells with the AcMNPV recombinant vAcCryIVD resulted in the expression of a protein with an M, of 80K (Fig. 6 and 7), which corresponded to the expected size of the truncated polyhedrin–CryIVD chimeric protein. In SDS–PAGE and immunoblot analyses of Sf cells infected with vAcCryIVD, this 80K protein was first detected about 24 p.i. and increased gradually in amount until about 72 p.i. (Fig. 7), at about which time most of the cells lysed. Replacement of most of the polyhedrin gene with the cryIVD gene in vAcCryIVD resulted in a concomitant loss of expression of the polyhedrin protein (Fig. 6 and 7). As the cells began to lyse, a small degree of proteolysis of the protein, yielding a slightly smaller peptide of 70K, was apparent in the Sf cultures (Fig. 7, lane 7).

The polyhedrin–CryIVD protein was expressed in the cytoplasm of the cells where refractile crystalline inclusions of the protein were visible by 40 h p.i. (Fig. 8d). By electron microscopy, most of the crystals were cubicoid and measured about 50 to 100 nm along the edge (Fig. 8e). The proteins assembled into the crystalline inclusions either in the soluble portion of the cytoplasm or in membrane-bound cisternae. No such inclusions were observed in control cells infected with AcMNPV vDA26Z or with LIGP-gal3, a polyhedrin-negative AcMNPV.

Properties of the polyhedrin–CryIVD protein produced in Sf cells and infected larvae

To determine whether the inclusions formed by vAcCryIVD in Sf cells had properties that corresponded with the native CryIVD protein produced by Bt, infected cells and purified inclusions were compared with native inclusions by proteolytic analysis with trypsin, and through bioassays against mosquito larvae.
AcMNPV expressing a mosquitocidal protein

Fig. 7. Time course of polyhedrin–CryIVD expression in Sf cells infected with the AcMNPV expression vector vAcCryIVD. (a) Results of SDS–PAGE; (b) results of immunoblotting experiments. Lane 1, M, markers; lanes 2 to 8, infected cells at 0, 16, 24, 32, 40, 48 and 72 h p.i., respectively; lanes 9, cells at 72 h infected with vDA26Z, a control recombinant AcMNPV with a full-length polyhedrin gene but which lacks the cryIVD gene.

Fig. 8. Light and electron micrographs of healthy Sf cells and cells infected with vAcCryIVD, a recombinant AcMNPV that expresses the Bt CryIVD protein fused to a truncated polyhedrin. (a) Healthy cells, (b) cells infected with wild-type AcMNPV, (c) cells infected with LIGP-gal3, a polyhedrin-negative AcMNPV control, and (d) cells infected with vAcCryIVD. The arrows point to clusters of crystals of the polyhedrin–CryIVD protein in the cytoplasm of the cell. (e) Electron micrograph of polyhedrin–CryIVD crystals in the cytoplasm of Sf cells infected with vAcCryIVD. Bar in (e) represents 200 nm.
Using a combination of differential centrifugation and sedimentation through sodium bromide gradients, it was possible to purify the crystalline inclusions containing the polyhedrin–CrylVD proteins from either infected cells or larvae. When sedimented through a 26 to 36% sodium bromide gradient for 90 min, the polyhedrin–CrylVD inclusions formed a single band about midway through the gradient, and this band corresponded in position to a band formed by CrylVD inclusions from Bt (Fig. 9). After washing and sedimentation, SDS–PAGE analysis of the contents of the band demonstrated that the protein inclusions in the preparation consisted almost exclusively of a single protein of 80K (Fig. 6, lane 5). In the immunoblot analyses, the polyclonal antibody raised against the CrylVD protein cross-reacted with the 80K protein observed in both the infected cells and preparations of purified inclusions, and with the native 72K CrylVD protein (Fig. 6b).

When solubilized and cleaved with trypsin, the 80K protein produced in Sf cells yielded major cleavage products of 33K and 40K, whereas the native CrylVD protein yielded products of 34K and 38K, all of which cross-reacted with the polyclonal antibody prepared against the native CrylVD protein (Fig. 6b).

Fig. 9. Sodium bromide gradient purification of polyhedrin-CrylVD crystals produced in Sf cells infected with the AcMNPV expression vector vAcCrylVD. Tubes illustrate the banding of crystalline inclusions of CrylVD protein purified from (a) Sf cells and (b) the natural CrylVD host, Bt. The arrow indicates the only band from the Sf cells that had significant mosquitocidal activity in bioassays.

Expression of the CrylVD protein in T. ni larvae

Fourth instars of T. ni infected by injecting larvae with non-occluded virions of vAcCrylVD from infected Sf cell cultures stopped feeding 3 days after infection, and all had died within a period of 5 days p.i. The protein composition of fat body from these infected larvae analysed by SDS–PAGE and immunoblotting also demonstrated the presence of an 80K protein that cross-reacted with the polyclonal antibody prepared against the CrylVD protein (Fig. 10). In slight contrast to the results obtained with vAcCrylVD-infected Sf cells, a lower amount of a smaller peptide of 70K, probably the result of proteolytic degradation, was also detected in the immunoblots of infected cells (Fig. 10b, lane 3). This apparent proteolytic degradation of the 80K protein was even more pronounced in the preparations of inclusions isolated from vAcCrylVD-infected larvae (Fig. 10, lane 5). In these, in addition to the 80K peptide, peptides of 36K and 70K were much more abundant, as detected by SDS–PAGE and immunoblotting, than in the inclusions from infected Sf cells (compare lanes 5 of Fig 6 and 10).

When preparations of the 80K inclusions isolated from vAcCrylVD-infected T. ni larvae were treated with trypsin, the results were also slightly different to those obtained with the preparations from infected Sf cells. Rather than two proteins of 34K and 40K as observed when Sf cell-derived inclusion preparations were treated with trypsin (Fig. 6, lanes 7 and 8), the trypsin-treated inclusion preparations from larvae consisted primarily of a peptide of 34K (Fig. 10, lane 7).

Biological activity of the polyhedrin–CrylVD chimeric protein

Cells infected with vAcCrylVD, whether from the Sf cultures or T. ni larvae, as well as the preparations of purified inclusions from these cells, exhibited toxicity to mosquito larvae (Table 1). The inclusion preparations were the most toxic and, irrespective of whether they were from Sf cells or fat body, were similar in toxicity, with LC50 values in the range of 225 to 250 ng protein/ml. The preparations of infected cells were approximately 10-fold less toxic, with the Sf cells exhibiting an LC50 of about 1.9 μg protein/ml, and the preparations from T. ni fat body an LC50 of 3.3 μg protein/ml.

Discussion

The results of this study show that the AcMNPV can be used to express and study the properties of mosquitocidal Bt proteins both in vivo and in vitro yielding information relevant to an understanding of the molecular biology of these proteins as well as to improvement of baculovirus insecticides. The results obtained with vAcCrylVD, the recombinant virus that expresses the full-length crylVD
gene, demonstrate clearly that a mosquitocidal polyhedrin-CryIVD protein with the expected size of 80K was produced in both infected Sf cells and T. ni larvae (Fig. 6 and 10, Table 1). The size and cuboidal shape of the inclusions produced in the cytoplasm of infected Sf cells and larvae were similar but not identical to those of native CryIVD inclusions, which typically are bar-shaped and measure about 60 x 150 nm in longitudinal section (Ibarra & Federici, 1986). This slight variation in size and shape may be due to the presence of the 57 amino acids of the polyhedrin added to the N terminus of the CryIVD protein.

Proteolytic cleavage of the CryIVD-C and CryIVD proteins produced using the recombinant baculoviruses was also similar to that obtained with the native CryIVD protein (Fig 4, 6 and 10). Some differences were observed in the size of the peptides apparently cleaved from the N terminus, for example in the case of the polyhedrin–CryIVD protein where larger amounts of a 40K protein were observed in comparison to that obtained by cleavage of the native protein (Fig. 6a, lanes 7 and 8). Again, the presence of the polyhedrin fragment at the N terminus of the recombinant proteins most likely accounted for these size differences.

Minor differences were also observed in the size and abundance of intact and trypsin-cleaved proteins depending on whether their source was Sf cells or larvae. Basically, the preparation of cells or purified inclusions from larvae appeared to be less stable than those from Sf cells (Fig. 6 and 10, lane 5). This is probably due to the presence of the larger amounts of proteases that occur in larvae and adhere to the inclusions during the purification process. A similar phenomenon is known to occur with other inclusions, such as polyhedra (McCarthy & Di Capua, 1979) and other chimeric proteins expressed in, and purified from, larvae (Kuroda et al., 1989; Maeda, 1987).

With LC<sub>50</sub> values around 250 ng/ml, the toxicity of the polyhedrin–CryIVD fusion protein was considerably less than that of the native protein, which has a toxicity, when assayed as purified inclusions under similar test conditions, of about 40 ng/ml (Ibarra & Federici, 1986). This apparently lower level of toxicity may not indicate an actual difference in toxicity, apart from the lower specific activity resulting from the presence of the polyhedrin fragment, but rather our inexperience in obtaining highly purified inclusions from Sf cells and larvae. Though the proteins in our purified inclusion preparations (Fig. 6 and 10) consisted primarily of the polyhedrin–CryIVD fusion proteins, other proteins were present, in addition to membrane fragments (data not shown). The presence of the latter may have had some effect on the ability of larvae to ingest the inclusions. Alternatively, lower toxicity could be due to conformational differences in the recombinant protein that altered toxin structure or processing in vivo.

One of the more interesting results obtained in our study was that the deletion of a 9-6K fragment at the N terminus of the CryIVD protein resulted in a total loss of toxicity, despite the occurrence of the dominant 33K to
34K peptide in the trypsin digests (Fig. 4). Explanations for this include the possibility that the 34K protein is not involved in toxicity, the 38K protein is the actual toxin, or that the 34K protein requires the presence of 38K protein to be toxic, forming a binary toxin as in \textit{B. sphaericus}. The deleted fragment contains a hydrophobic domain, and the loss of toxicity may indicate that this domain is important to binding the toxin to the mosquito midgut. Alternatively, the full peptide may be required for proper folding of the protein, which once folded can be cleaved to yield a mosquitocidal peptide of 34K. These possibilities are currently being examined using the present baculovirus expression vectors as well as Bt–\textit{E. coli} shuttle vectors that have recently become available for expressing Bt proteins in crystal-minus mutants.

Whether Bt proteins will be useful for improving the efficacy of NPV insecticides remains to be determined. In two recent studies (Martens \textit{et al.}, 1990; Merryweather \textit{et al.}, 1990) recombinant AcMNPVs were used to express CryIA proteins active against lepidopterous insects in Sf cells. In both studies evidence was presented indicating the products were similar to the native proteins with respect to both the size of the protein and toxicity to lepidopterous larvae. In neither case did it appear that the Bt protein resulted in increased virulence of the recombinant viruses. The same is apparently true for our CryIVD virus. However, in all three studies the recombinant viruses were engineered to express protoxins rather than activated endotoxins. Before Bt toxins can be ruled out as candidates for improving baculovirus efficacy, attempts should be made to express activated toxins, as they occur in native toxins and fused with signal sequences that permit them to be secreted into the haemolymph.

Though it has not been considered previously, the baculovirus expression system might prove useful for studies of Bt's mode of action. The toxic action of Bt in \textit{vivo} is known to be dependent on the binding of activated toxin to the external surface of midgut microvilli, where the toxins appear to bind to specific proteins (Hofmann \textit{et al.}, 1988) and then intercalate, forming transmembrane cation pores (Knowles & Ellar, 1987) that lead to cell death. However, it has been shown recently that the CryIA and CryIIIA toxins can insert into planar lipid bilayers that have no protein receptors (Slatin \textit{et al.}, 1990). Moreover, recent evidence from patch clamp studies of the action of the CryIC toxin on Sf cells indicates that this Bt toxin may act inside the cell and is capable of inserting into the cell membrane from the cytoplasmic side (Schwartz \textit{et al.}, 1991). Thus, by using a baculovirus expression vector, it should be possible to circumvent the midgut microvillus barrier and express different forms of Bt toxins within cells, and to determine whether they are toxic and, if so, where they act. Such studies, providing they use activated toxin, might enable the intoxication mechanism to be separated from the binding action of the molecule and clarify whether specificity is determined solely at the level of the midgut epithelium. To some extent we attempted this in the present study by expressing in lepidopteran cells a toxin normally toxic only to mosquitoes. We might have expected some toxicity as it has been shown that the activated form of the CryIVD toxin is toxic (LC\textsubscript{50} 94 ng/ml) to \textit{M. brassicaceae} cells in \textit{vivo} (Chilcott & Ellar, 1988), and some proteolysis of Bt occurs in cell cultures and larvae (present study and Merryweather \textit{et al.}, 1990). However, our present results are inconclusive with respect to the toxicity of any activated proteins that may have been released inside the cell, because the CryIVD protein crystallized into inclusions, apparently soon after synthesis, and the amount of proteolysis was minimal.

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References


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