Characterization of \( v \)-cath, a cathepsin L-like proteinase expressed by the baculovirus \textit{Autographa californica} multiple nuclear polyhedrosis virus

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\textit{Autographa californica} multiple nuclear polyhedrosis virus (AcMNPV) contains a 966 bp ORF that encodes a papain type cysteine proteinase with cathepsin \( L \)-like characteristics. Using Western blot analysis of infected cell extracts we showed that \( v \)-cath proteinase has 35.5 kDa and 32 kDa precursor forms which are processed to a 27.5 kDa mature form in a manner characteristic of papain and cathepsin \( L \). \( V \)-cath proteinase activity was greatest under acidic conditions (pH 5.0) and was reduced in the presence of the cysteine proteinase inhibitors, leupeptin and E64. Urea, a known enhancer of cathepsin \( L \) activity, also enhanced \( v \)-cath proteinase activity. AcMNPV \( v \)-cath proteinase was detected post-mortem in tissues of insects infected with wild-type (wt) virus. Insects infected with a \( v \)-cath deletion mutant did not become flaccid after death as is normally observed with wt AcMNPV infections. These findings indicate a link between \( v \)-cath activity and degradation of host tissues during virus pathogenesis.

Introduction

Baculoviruses are a family of arthropod specific viruses; most species have been isolated from insects, some of which are significant agricultural or forest pests. A potential for use of native baculoviruses as bioinsecticides is yet to be fulfilled due in part to the narrow host range and low virulence of the viruses; however, efforts are underway to create novel strains by regulating viral genes and by incorporating exotic genetic material into the native virus. The baculovirus \textit{Autographa californica} multicapsid nuclear polyhedrosis virus (AcMNPV) has been studied extensively as both a foreign protein expression vector and as a bioinsecticide. A 966 bp ORF located between 79.8 and 81.1 m.u. on the AcMNPV genome was found in 1991 during nucleotide sequence analysis of the region downstream of the gp67 gene (Whitford \textit{et al.}, 1989). This ORF had an apparent homology to papain family cathepsin proteinases and was submitted to GenBank (accession number M67451) as \textit{viral-cathepin} or \( v \)-cath. A \( v \)-cath gene has also been identified in the genome of \textit{Choristoneura fumiferana} nuclear polyhedrosis virus (CfNPV) (Hill \textit{et al.}, 1992). The calculated molecular mass of \( v \)-cath protein is 37 kDa and Cys\textsuperscript{136} and His\textsuperscript{268} have been proposed as \( v \)-cath proteinase catalytic site residues. A \textit{Bombyx mori} nuclear polyhedrosis virus (BmNPV) proteinase having 96.3% amino acid sequence identity to \( v \)-cath has recently been reported (Ohkawa \textit{et al.}, 1994). Global amino acid sequence alignments were employed by the authors to suggest that this \( v \)-cath homologue had highest identity to a proteinase of the protozoan \textit{Trypanosoma brucei} (Lonsdale-Eccles &Grab, 1987). Close examination of the catalytic site regions of \( v \)-cath using specific alignment and examination of the size of the peptide (Fig. 1) suggest it is significantly more related to mammalian cathepin \( L \) and a lobster cysteine proteinase, LCP1 (Laycock \textit{et al.}, 1991). Based on comparisons with papain, Pro\textsuperscript{113} of the \( v \)-cath amino acid sequence is a probable post-translational cleavage site. The amino acid sequence of \( v \)-cath protein also has the potential N-glycosylation motif (Asn–X–Ser) at residues Asn\textsuperscript{58} and Asn\textsuperscript{104}.

In this study we present evidence for expression of cathepin \( L \)-like cysteine proteinase activity in cells infected with AcMNPV and show that \( v \)-cath proteinase may be processed and compartmentalized in a similar manner to mammalian cathepsins. We also report that \( v \)-cath proteinase expression may have significant effects on pathogenesis of infection \textit{in vivo}.

Methods

Cells lines and viruses. \textit{Spodoptera frugiperda} (SF-21) cells (Vaughn \textit{et al.}, 1977) were cultured at 28 °C in BML-TC/100 insect tissue culture medium (Gardiner &Stockdale, 1975) supplemented with 10% fetal calf serum and 50 \( \mu \)g/ml gentamicin sulphate. AcMNPV strain HR3 (Brown \textit{et al.}, 1979) is designated wt (wild-type) in the text. AcMNPV
strain BacPAK6 (Kitts & Possee, 1993) is a pol(-) derivative of AcMNPV C6 (Possee et al., 1991). We used it for construction of v-cath over-expression mutants (cath + AcMNPV). All virus types were used at an m.o.i. of 0.1 to 0.2 for preparation of non-occluded virus stocks (Faulkner & Henderson, 1972), and at an m.o.i. of 10 for metabolic procedures.

Construction of recombinant virus with altered v-cath activity. A v-cath over-expression mutant of AcMNPV was created using a PCR. Two oligonucleotide primers were constructed to correspond to the opposite ends of the v-cath gene (5' TAAGGATCCCCCGGGTAAGTTGTAA-TATTATTAGTGT3', 5' TAAGGATCCCTAGGACGAGTGTGTT-GAGATTATA 3'). The 5' ends of each primer incorporated restriction endonuclease (REn) sites so that the amplified v-cath gene had a unique Smal site at one end and a unique XhoI site at the other end to permit direct cloning of the v-cath gene into the AcMNPV transfer vector pBacPAK9 (Kitts & Possee, 1993) and to produce pBacPAK/v-cath. In the construct, the v-cath gene lies downstream of and in the same orientation as a polyhedrin promoter. pBacPAK/v-cath DNA was co-transformed with Bsa36l REN cut BacPAK6 AcMNPV DNA into Sf cells as described by Kitts & Possee (1993). Recombinant virus was isolated by standard plaquing methods (Brown et al., 1989) was ligated to produce the insertional mutation within the v-cath gene. The viral clone AcDCV1, containing the cassette in a orientation as AcMNPV in the text.

Expression of v-cath as a bacterial fusion protein. The 2.4 kbp BamHI-E fragment of AcMNPV DNA was digested with EcoRI and PstI RENs to produce a 567 bp DNA fragment representing the C terminus region of the 966 bp v-cath gene. This EcoRI-PstI v-cath fragment was cloned in frame with the maltE gene of the bacterial fusion protein expression vector pMAL-c2 (New England Biolabs). The resulting MBP–v-cath (EcoRI/PstI) fusion protein was expressed in Escherichia coli (JM109) and consisted of a 23 kDa N-terminal, maltE gene product (maltose binding protein; MBP), and a 23 kDa C-terminal v-cath protein (C terminus region of v-cath). The fusion protein was purified on an amylase resin affinity column and the identity of the fusion protein was verified by treatment with the factor Xa protease (New England Biolabs) which cleaved MBP from the v-cath (EcoRI/PstI) protein allowing separation on a 10% SDS-PAGE gel.

Cell lysates. Sf cells infected with AcMNPV cultured in 75 cm² tissue culture flasks were dislodged by scraping and suspended in culture media. Suspended cells were pelleted (5 min at 1000 g) and resuspended at 4 °C in 200 μl volumes of lysis buffer [10 mM-NaPO₄, 10 mM-EDTA, 10 mM-cysteine and 10% (w/v) glycerol, pH 6.5] and were lysed by sonicating (Branson F-Module) for 30 s and by shearing using a micropipette. Lysates were centrifuged (5 min at 15,000 g) and the supernatant fractions (S15) were collected. Pellet fractions (P15) were resuspended in 200 μl volumes of lysis buffer and 1% (w/v) Triton X-100.

Polycional antibodies to the v-cath peptide. MBP–v-cath (EcoRI/PstI) fusion protein [100 μg (250 μl)] was combined with 100 μg of QuilA adjuvant (250 μl). Immunization of rabbits was done as described elsewhere (Harlow & Lane, 1988). Serum was tested by Western blotting and the background effect was reduced by pre-absorbing with MBP and E. coli antigens.

Protein gels. Protein samples were denatured by boiling in 0.05 M-Tris–HCl pH 6.8, 2% SDS, 5% mercaptoethanol and were fractionated in 12% polyacrylamide SDS–PAGE gels (Laemmli, 1970). Pre-stained protein molecular mass markers (Bio-Rad) were run alongside samples. Protein samples were also separated under non-denaturing conditions in 6% polyacrylamide non-equilibrium pH gel electrophoresis (NPEPGE) gels (O'Farrell et al., 1977). Pre-stained isoelectric focussing (IEF) markers (Bio-Rad, pH 4-7 to 9.6) were run alongside NPEPGE gel samples.

Western blotting. Western blot analysis of v-cath protein was done using the ECL Western blotting detection reagent kit (Amersham). Cell lysates were harvested, electrophoresed and then transferred to nitrocellulose. The nitrocellulose blot was blocked for 12 h in 10% (w/v) skim milk, PBST solution (10 mM-PO₄, 120 mM-NAcI, 2.7 mM-KCl and 0.02% ,w/v, Tween-20, pH 7.4). Following blocking, the blot was treated for 2 h with specific antigen at a 1:700 dilution in PBST. After three washes in PBST, the blot was treated for 2 h with goat anti-rabbit alkaline phosphatase conjugate antiserum at a 1:2000 dilution in PBST. Following three more washes in PBST, reagent 1 (100 mm-
Tris-HCl pH 8, 5 mM-H,0, and reagent 2 (2 mg-luminol, 78 µm-luciferin) of the Amersham ECL system were combined in equal proportions and reacted with the blot. The blot was exposed to X-ray film (Kodak X-Omat) for 10 to 15 s.

Proteolytic assays with azocasein. The assay was done using cell extracts and was an adaptation of a method described by Riemann et al. (1982). Cell lysates were incubated in azocasein substrate solution (0.2%, w/v, azocasein, 3 M-urea, 5 mM-cysteine, 5 mM-EDTA and 50 mM-citrate, pH 5.4). Samples of substrate solution (600 µl) were preheated to 37 °C prior to addition of 10 µl (50 µg protein) volumes of cell lysates. The mixture was incubated at 37 °C for 100 min, then TCA was added to 10% w/v. Following centrifugation in a microfuge (5 min, 15000 g), A405 of the supernatants was recorded.

Dilutions (1:150) of a papain (554 U/ml, Sigma, 2 x crystallized papain suspension in 0.05 M-sodium acetate, pH 4.5, 0.01% thymol) were made in lysis buffer and 1 to 10 µl volumes were incubated in azocasein substrate solutions. A standard curve was generated relating caseinolytic activity (A405) to the known unit quantity of papain. One papain unit (U) is defined as the activity required to degrade 1 µmol of BAEE (N-x-benzoyl-l-arginine ethyl ester)/min at pH 6.2 at 25 °C.

When proteinase inhibitors were employed in experiments, 1 mM concentrations of aprotinin, leupeptin or E-64 were mixed 1:1 with cell lysates and incubated for 20 min at room temperature prior to proteolytic assays with azocasein.

Proteinase detection using X-ray film. The method is based on the observation that proteolytic activity causes the gelatin coating on X-ray film to be degraded (R. St Leger, personal communication). Cell lysates were separated in acrylamide gels by the NEPHGE method and Kodak X-Omat X-ray film which had been soaked in 5 mM-cysteine, 3 M-urea, 5 mM-EDTA and 50 mM-citrate (pH 5.4) was overlaid onto the gel. Incubation was for 30 min at 25 °C followed by washing of the film in warm water.

In vivo experiments. Trichoplusia ni fifth instar larvae were provided by the Boyce Thompson Institute for Plant Research, Ithaca, NY, USA. Larvae were injected at the hind-most proleg with 10 µl of non-occluded viral inoculum (diluted in cell culture medium to contain 1 x 10^6 TCID, units of virus) using a 26 gauge needle. Insects were observed over the course of the infection and were photographed approximately 2 days after death (7 days post-inoculation).

Results

Immunodetection of v-cath protein in insect cells

To determine the specificity of anti-v-cath PAb sera, Western blots were done on proteins from Sf cells infected with mutated strains of AcMNPV (Fig. 2). The v-cath protein was not detected in cells infected with cath− AcMNPV nor in mock infected cells. The 27 kDa v-cath mature peptide was made in cells infected with either wt AcMNPV or the pol(−) BacPAK6 derivative, cath+ AcMNPV; 32 kDa and 35.5 kDa peptides were also detected in cells infected with either wt or cath+ AcMNPV but were more prominent in cells infected with cath+ AcMNPV. Preliminary investigations were done to investigate the relationships between the three v-cath peptide species detected by the antiserum. Enhanced accumulation of 27.5 kDa v-cath peptide relative to the 32 kDa and 35.5 kDa peptides was seen in lysates from cath+ AcMNPV-infected cells (Fig. 3, lane 2). The presence of tunicamycin in growth media increased accumulation of the 32 kDa v-cath species and an additional 24 kDa peptide (Fig. 3, lane 3).

A time course for production of v-cath protein was done by Western blot analysis of extracts from infected cells. In addition cell lysates were fractionated into S15 and P15 fractions to localize v-cath into supernatant and pellet components: v-cath protein was detected primarily in the P15 fractions of cells infected with either cath+ or cath− AcMNPV.
wt AcMNPV (Fig. 4). Although the relative levels of \( v\text{-cath}\) protein expression were higher in cells infected with \( c\text{ath}^+\) AcMNPV, expression occurred at later times in both \( c\text{ath}^+\) and wt AcMNPV virus infections (22 h–56 h p.i.). In addition, 32 kDa and 35.5 kDa \( v\text{-cath}\) protein species were detected after the 27.5 kDa protein was first seen; \( v\text{-cath}\) protein species were not detected in cells infected with the deletion mutant \( c\text{ath}^-\) at any time.

Detection of cathepsin-like proteinase activity in lysates of insect cells

The amino acid sequence of \( v\text{-cath}\) suggested that it was a proteinase; thus enzyme activity was assayed in lysates from infected cells. Lysates from cells infected with \( c\text{ath}^+\) AcMNPV had at least double the enzyme activity of extracts from cells infected with either wt virus or the \( pol(\sim)\) BacPAK6 recombinant (Fig. 5a). The lowest caseinolytic activities were detected in lysates of \( c\text{ath}^-\) AcMNPV and mock infected cells. Cells infected with \( c\text{ath}^+\) AcMNPV were found to split the substrate azocasein at a high rate which was greatest at pH 5.0 and 4- to 5-fold enhanced by the addition of urea to the assay mixtures (data not shown).

The serine protease inhibitor aprotinin and the cysteine proteinase inhibitors leupeptin and E64 were employed to help define the class of proteinase activity measured in the cell lysates. Aprotinin had little effect on enzyme activity while leupeptin and E64 diminished the activity significantly (Fig. 5b).

To further strengthen the correlation of proteinase activity with \( v\text{-cath}\) protein expression a time course assay for caseinolytic activity was done (Fig. 6). A graded response was seen by analysis of lysates from cells infected with wt or \( c\text{ath}^+\) virus but no increased activities were measured in lysates from cells infected with the deletion mutant \( c\text{ath}^-\) AcMNPV. Caseinolytic activity was easily detected in P15 fractions of cells infected with both wt and \( c\text{ath}^+\) AcMNPV strains but was greatest in those from \( c\text{ath}^+\) AcMNPV infected cells.

A gelatin clearing technique was used to detect
v-cath, a baculovirus proteinase

Fig. 6. Proteinase accumulation and distribution in cell extracts. Cells were infected with cath+ (■), wt (■) or cath− (□) strains of AcMNPV and harvested at intervals up to 56 h p.i. Lysates were prepared, fractionated by centrifugation into pellet (P15) and supernatant (S15) components and assayed for proteinase. Enzyme activities in pellet fractions are shown in (a) and in the extract supernatants in (b). Error bars indicate 1 SD above the mean.

proteases after IEF of cell peptides (Fig. 7). Gel lanes loaded with lysates from cells infected with wt or cath+ virus caused gelatin clearing on the X-ray film at a zone corresponding to pl 5 while a lane loaded with papain (pl = 8.8) cleared on the film at the pl 9 zone. No proteolytic activity was observed when gel lanes were loaded with lysates from cells infected with cath− or from mock infected cells.

Effect of v-cath gene modification on the pathogenesis of AcMNPV infection

The external appearance and pattern of pathogenesis was different from that seen in wt infections when fifth instar T. ni larvae were infected with cath+ and cath− recombinant virus (Fig. 8). Larvae injected with wt virus died in about 5 days and became progressively flaccid; their cuticle darkened and was black within a day after death. Internal tissues of the cadavers were liquified and laden with occlusion bodies. The 27.5 kDa v-cath mature peptide was identified by Western blot analysis in the liquified remains of larvae fed with wt virus and persisted for at least 2 days after death (results not shown). Insects injected with cath− AcMNPV took on a bleached

Fig. 7. Comparison by pl measurement of v-cath and papain. Extracts were made at 48 h p.i. from infected cells. Proteins were separated by IEF on a 6% polyacrylamide NEPHGE gel. After washing an X-ray film was overlaid onto the gel. Gelatin degradation on the film is shown (white regions). Cells were infected with cath− (lane 1), wt (lane 2) or cath+ (lane 3) AcMNPV. A mock infected cell lysate (lane M) and papain (lane P) were also focused on the same gel.

Fig. 8. Trichoplusia ni larvae are shown 2 days post mortem and 7 days post viral inoculation (1 × 10⁵ TCID₅₀ units). The control group of larvae which survived to pupation were mock injected with the media in which viral inoculums had been diluted. The cath+ virus infected larvae turned black 1 day after the wt virus group of insects. The wt virus infected larvae were extremely fragile and the bodily contents of the upper wt larvae have spilled out. The cath− infected larvae were pale white and contrasted sharply from the lime-green colouration of the original healthy larvae.
whitish appearance and internal tissue integrity was maintained after death. Cuticles of larvae infected with \textit{cath} + \textit{AcMNPV} often remained light-brown after death and the degree of tissue liquefaction also appeared to be reduced.

**Discussion**

Evidence that the baculovirus \textit{v-cath} gene expresses cysteine proteinase activity is based on measurements of substrate specificity, reaction conditions and sensitivity to protease inhibitors (Figs 5 and 6). The pH optimum (5.0) of the enzyme on azocasein and the enhancement of activity in the presence of 3 M-urea are indicative of a cathepsin L-like proteinase (Barrett & Kirschke, 1981; Wiederanders \textit{et al.}, 1987). Selective inhibition by cysteine proteinase inhibitors also fits the premise that \textit{v-cath} is a cathepsin L-like cysteine proteinase (Fig. 5b). Rawlings \textit{et al.} (1992) were unable to detect enhanced cysteine proteinase activity in lysates from cells infected with \textit{AcMNPV}. Possibly the fluorescent peptide substrate, Z-Phe-Arg-NHMec, used by them was not a preferred substrate of baculovirus \textit{v-cath}. Peptide substrate assays are commonly subject to light scattering and quenching effects when carried out with unpurified cellular lysates (Sarath \textit{et al.}, 1989; Barrett & Kirschke, 1981). The substrate azocasein used in this study has been reported not to react with background interfering substances (Barret & Kirschke, 1981). Assuming \textit{v-cath} is formed by post-translational cleavage at Pro\textsuperscript{113}, the calculated pI of the mature 27.5 kDa \textit{v-cath} protein is 5.0; the X-ray film assay confirmed this pI value for the proteinase activity detected in cell lysates (Fig. 7).

The 35.5 kDa and 32 kDa \textit{v-cath} protein species which were prominent when \textit{v-cath} was over-expressed (Fig. 3) are likely precursors of a 27.5 kDa mature form of \textit{v-cath}. Precursor forms (41 kDa and 36 kDa) of 24.5 kDa papain were reported when the enzyme was made using a baculovirus expression vector system (Vernet \textit{et al.}, 1990). A model for the expression and processing of \textit{v-cath} is proposed in Fig. 9. Results of N-glycosylation inhibition using tunicamycin support the premise that the 32 kDa \textit{v-cath} protein species is N-glycosylated to form the 35.5 kDa protein species and that a 24 kDa \textit{v-cath} protein species is a non-glycosylated form of the mature 27.5 kDa \textit{v-cath} species. N-Terminal cleavage of non-glycosylated \textit{v-cath} at the predicted site, Pro\textsuperscript{113} would produce a 24 kDa cleavage product. The evidence suggests that N-glycosylation occurs at Asn\textsuperscript{158} on the \textit{v-cath} protein amino acid sequence. By analogy to a previously described model of papain processings (Vernet \textit{et al.}, 1990) the 35.5 kDa and 32 kDa \textit{v-cath} protein species are likely glycopro-v- \textit{v-cath} and pro- \textit{v-cath} intermediates which are cleaved to form a 27.5 kDa mature \textit{v-cath} protein (Fig. 9). It should be noted that a 27 kDa cysteine proteinase has been purified from the extracts of BmMNPV infected Sf-9 cells (Ohkawa \textit{et al.}, 1994). BmMNPV has 95\% identity to 90\% of AcMNPV sequences (Majima \textit{et al.}, 1993) and is likely to contain a very similar \textit{v-cath} gene homologue.

We studied the temporal expression and cellular localization of \textit{v-cath} protein. Transcriptional analysis indicated a delayed baculovirus late promoter region having the TAAG motif is located upstream (−29 to −25) of the \textit{v-cath} reading frame and is the likely start site of \textit{v-cath} gene expression (data not shown). Data from Western blots (Fig. 4) and proteinase assays (Fig. 6) confirm a delay in enzyme detection until at least 22 h p.i. \textit{v-cath} proteinase was found associated predominately in P15 fractions (Fig. 4) which contain nuclei, mitochondria and lysosomes (DeDuve, 1975). The cathepsin-like features of \textit{v-cath} protein suggest it may be compartmentalized into lysosomal organelles. Expression of \textit{v-cath} late in infection may contribute to liquefaction of tissues observed in pathogenesis. Liquefaction of insect tissues during AcMNPV infection could be considered as somewhat analogous to stages in lepidopteran moulting and metamorphosis. During the ecdysis phase of moulting, when insects shed their outer cuticle, and during metamorphosis, when lepidopteran larval structures change to adult structures, auto-lysosomal proteinases are released which break down and liquify insect tissues (Low \textit{et al.}, 1993; Komuves \textit{et al.}, 1985). No \textit{v-cath}-like cathepsins have been reported to be associated with either moulting or metamorphosis in lepidopterans, but a cathepsin B-like proteinase and a chitinase have been
found in moulting crabs (Samuels & Renolds, 1993; O'Brien & Skinner, 1988). A chitinase gene lies adjacent to the v-cath gene in the genome of AcMNPV (Hawtin et al., 1992). We considered it is possible that both v-cath and the viral chitinase participate in the destruction of insect tissues during the later stages of pathogenesis. In baculovirus infections v-cath expression may be linked to late expression of v-ubi (Guarino, 1990), an AcMNPV gene encoding for a ubiquitin-like peptide. Insect ubiquitin is expressed at high levels during the maturation of auto-lysosomes in Spodoptera frugiperda cells (Low et al., 1993). A role for baculovirus v-ubi may be to enhance the maturation of v-cath present within auto-lysosomal organelles.

Observation of infected insects (Fig. 8) suggests that v-cath also induces melanization by triggering a cascade of events leading to activation of pro-phenoloxidases. Melanization is a process required in cuticle sclerotization and haemolymph clotting (Lai-Fook, 1966). Pro-phenoloxidases are known to be activated in the presence of proteolytic enzymes (Dohke, 1973) and their uncontrolled premature activation may have fatal consequences for an insect (St Leger et al., 1988). The reduced level of blackening seen in insects infected with catt + AcMNPV may be caused by the insolubility of over-expressed v-cath enzyme or highly localized activation of the phenoloxidase system leading to walling off and isolation of infected larval tissues.

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