

A Virus Essential for Insect Host-Parasite Interactions Encodes Cystatins

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***Cotesia congregata* is a parasitoid wasp that injects its eggs in the host caterpillar *Manduca sexta*. In this host-parasite interaction, successful parasitism is ensured by a third partner: a bracovirus. The relationship between parasitic wasps and bracoviruses constitutes one of the few known mutualisms between viruses and eukaryotes. The *C. congregata* bracovirus (CcBV) is injected at the same time as the wasp eggs in the host hemolymph. Expression of viral genes alters the caterpillar's immune defense responses and developmental program, resulting in the creation of a favorable environment for the survival and emergence of adult parasitoid wasps. Here, we describe the characterization of a CcBV multigene family which is highly expressed during parasitism and which encodes three proteins with homology to members of the cystatin superfamily. Cystatins are tightly binding, reversible inhibitors of cysteine proteases. Other cysteine protease inhibitors have been described for lepidopteran viruses; however, this is the first description of the presence of cystatins in a viral genome. The expression and purification of a recombinant form of one of the CcBV cystatins, cystatin 1, revealed that this viral cystatin is functional having potent inhibitory activity towards the cysteine proteases papain, human cathepsins L and B and *Sarcophaga* cathepsin B in assays in vitro. CcBV cystatins are, therefore, likely to play a role in host caterpillar physiological deregulation by inhibiting host target proteases in the course of the host-parasite interaction.**

Parasitoid wasps are insects that parasitize other insects, resulting in the death of the host (52). One of the most original strategies developed by these wasps is the disarmament of their hosts' immune system by injection of polydnviruses (PDVs) during oviposition (7, 9, 62). PDVs are divided in two genera, ichnoviruses and bracoviruses, and are associated with tens of thousands of species of endoparasitoid wasps belonging to two different families, ichneumonids and braconids, respectively (24). The wasp-PDV association is one of the rare examples of mutualism between eukaryotes and viruses (74), in addition to viruses involved in human placenta organogenesis (48). Viral DNA is integrated into the genome of the wasp and is transmitted vertically via the wasp chromosomes (12, 18, 25, 29, 58, 77). Virus excision and replication occur only in the wasp ovaries, initiated during maturation to adult tissue and resulting in the production of a segmented genome composed of numerous double-stranded DNA circles, which are encapsidated singly or in groups (3, 72). Virus particles are injected

into the insect host where viral gene expression occurs ensuring parasitism success (72).

Cotesia congregata (Microgastrinae) is a braconid endoparasitoid wasp of the tobacco hornworm caterpillar, *Manduca sexta*. The *C. congregata* bracovirus (CcBV) is strongly implicated in many of the parasitism-induced effects observed in the parasitized host including a pronounced change in the host's developmental program, such as developmental arrest at the last instar, alteration of host immunity, inhibition of feeding, and induction of pigmentation changes (10). Recently, the entire genome of CcBV was sequenced and annotated, revealing the presence of numerous putative genes possibly involved in host physiological deregulation (22). Many of these genes are organized in families with the largest family comprising protein tyrosine phosphatases (51). In this report, we present the characterization of a novel CcBV multigene family encoding three proteins, cystatins 1 through 3, with significant homology to type 2 cystatin members of the cystatin superfamily of proteins. Type 2 cystatins are natural, tightly binding, reversible inhibitors of cysteine proteases belonging mainly to the C1 family of peptidases (53). In recent years, new members of the superfamily have been characterized, including phyto-cystatins and insect cystatins (1, 2, 15, 53).

Other cysteine protease inhibitors, such as inhibitors of apoptosis proteins, have been described in virus genomes (16, 33), but here we present the first report on the characterization of

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genes in a virus encoding cystatins. We show that one of the CcBV cystatins is a functional inhibitor of cysteine proteases of the C1 family. The putative targets of these viral cystatins and their possible involvement in host physiological deregulation are discussed.

MATERIALS AND METHODS

Insect rearing. The parasitic wasps (*C. congregata*; Hymenoptera, Braconidae, Microgasterinae) were reared on their natural host, the tobacco hornworm, *M. sexta* (Lepidoptera, Sphingidae) as previously described (31, 50).

DNA and RNA extractions. Virus DNA for plasmid genomic library construction, field inversion gel electrophoresis (FIGE), and Southern blot analysis was prepared following purification of virus particles by filtration, as previously described (10, 31). For sequencing, viral DNA was extracted from 1,000 female wasp ovaries and amplified by using the rolling circle DNA polymerase TempliPhi (Amersham Biosciences). CcBV double-stranded DNA circles were sequenced by a shotgun strategy (22).

Total RNA was extracted from 3 to 20 fourth-instar *M. sexta* larvae parasitized by *C. congregata* at different time intervals after parasitoid oviposition (two ovipositions were checked visually) and from synchronous nonparasitized controls. Extractions were performed on fat body or midgut with the QIAGEN RNA/DNA maxi or midi kit according to the manufacturer's instructions. Nerve cord and brain (nerve chain) or Malpighian tubules were stored in RNAlater solution (QIAGEN, France) after dissection; when sufficient material was obtained, extractions were performed using the QIAGEN RNeasy Plant Mini kit. Hemocytes were collected by centrifugation at $300 \times g$ for 5 min, and RNA was extracted using the RNeasy Plant Mini Kit. Poly(A)⁺ mRNAs were selected with the QIAGEN Oligotex mRNA midi kit. Wasp RNA was extracted with the RNeasy Plant Mini Kit.

FIGE analysis. Two hundred and fifty nanograms of virus DNA was separated at 4°C by FIGE (Bio-Rad, France) as previously described (51). Separated virus DNA circles were transferred onto nylon membranes (Biotrans ICN, France) in 0.4 N NaOH as described in the manufacturer's protocol. Hybridization was carried out overnight at 65°C using a labeled cystatin 1 cDNA fragment as probe. Membranes were washed twice in $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 20 min and with $0.2 \times$ SSC–0.1% SDS for 20 min at 65°C before autoradiography.

Preparation and screening of CcBV genomic and cDNA libraries. EcoRI, HindIII, and EcoRI-HindIII fragments of the CcBV genome were cloned into the plasmid pBluescript KS by standard procedures (57). DNA minipreplications of individual clones were screened by a reverse Northern approach, using ³²P-labeled first-strand cDNA probes synthesized from poly(A)⁺ mRNA isolated from *M. sexta* 24 h after parasitoid oviposition. One microgram of poly(A)⁺ RNA was reverse transcribed into first-strand cDNA using Superscript II RNase H- Reverse Transcriptase (Life Technologies, France). The single strand cDNA was then labeled by standard procedures (57).

A cDNA library was constructed from total RNA isolated from *M. sexta* fourth-instar larvae 24 h after parasitization, using a SMART cDNA library construction kit (Clontech) according to the manufacturer's instructions. The library was screened using the 6-kb-long EcoRI fragment from the E6 CcBV genomic DNA clone previously isolated by reverse Northern techniques by standard methodology (57). Individual positive plaques from the secondary screening were isolated in agar plugs, eluted in suspension medium (SM; 0.1 M NaCl, 0.01 M MgSO₄ · 7H₂O, 0.35 M Tris [pH 7.5], 0.01% gelatin) buffer, and then converted to pTriplex2 in *Escherichia coli* (BM25.8) by in vivo excision and circularization to form a complete plasmid from the recombinant phage. DNA sequencing reactions of selected clones were carried out using the ABI PRISM BigDye Terminator V3.0 Sequencing kit (Applied Biosystems, France) according to the manufacturer's instructions on an ABI PRISM 3100 Avant sequencer (Applied Biosystems, France). DNA and protein sequences were subjected to computer BLAST analysis (4).

Southern and Northern blot analysis. Two hundred nanograms of viral DNA was electrophoretically separated on a 0.8% Tris-acetate-EDTA agarose gel. Twenty micrograms of RNA was electrophoretically separated on a 1.2% Tris-acetate-EDTA nondenaturing agarose gel (70). Separated DNA or RNA was transferred onto a nylon membrane (Biotrans ICN, France) in 1.5 M NaCl–0.5 M NaOH or in $20 \times$ SSC, respectively, as described in the manufacturer's protocol. The filters were hybridized overnight at 65°C with a labeled cDNA cystatin 1 fragment probe or actin probe. A 833-bp fragment from the *Manduca sexta* actin gene (GenBank accession number L13764) was amplified using primers Actine 1 (5'-CTGGGATGACATGGAGAAGA-3') and Actine 2 (5'-ACATCT

GCTGGAAGGTGGAC-3') and used as a probe in control experiments. Membranes were washed once in $2 \times$ SSC–0.1% SDS for 20 min and once in $0.2 \times$ SSC–0.1% SDS for 20 min at 65°C before autoradiography. The intensities of radioactive signals were measured on membranes with an Instant Imager (Packard, France). Northern blots were repeated at least twice with the same RNA extracts and gave similar results.

Production of recombinant cystatin 1 protein in insect cells. The expression cassette pIE1/153A (23, 45) was employed for expression of recombinant cystatin 1 in insect cells. Plasmid pIE1/153A.cyst was generated as follows. Primers CystF (5'-TCGAGCGGCCGCAATGGGCAAGGAATATCGAG-3') and CystR (5'-TGGCGCGGCCGCTTAACAATTTTCATATCCCAAC-3') containing the first 19 and last 23 bases of the cystatin 1 gene, respectively, with extensions for NotI restriction sites (underlined), were used in a PCR amplification reaction using *Pfu* polymerase (Promega) and the relevant pTriplex2 clone as a template. The reaction (35 cycles, each consisting of denaturation at 94°C for 40 s, annealing at 50°C for 40 s, and extension at 72°C for 80 s) yielded a product containing the cystatin 1 open reading frame (ORF) flanked by NotI restriction sites. This was digested and cloned in the unique NotI site of pBluescript (Stratagene) by standard procedures (57). After sequence verification, the fragment was subcloned into the unique NotI site of pIE1/153A to yield pIE1/153A.cyst.

BTI-TN-5B1-4 cells (HighFive; Invitrogen) adapted to ESF 921 protein-free insect cell culture medium (Expression Systems, Inc.) were routinely subcultured weekly at 28°C. Transfection of insect cells using Lipofectin reagent (Invitrogen) was performed using an optimized procedure as previously described (36). In brief, 5×10^5 cells/ml in 2 ml of medium were used. The medium was replaced by a transfection solution that contained 30 µg/ml Lipofectin and 5 µg/ml plasmid DNA. After 5 h, the transfection solution was replaced by fresh medium. For the generation of stably transformed cell lines, HighFive cells were cotransfected with pIE1/153A.cyst and pBmA.PAC, a plasmid conferring resistance to puromycin in lepidopteran cells (P. Farrell and K. Iatrou, unpublished results), at molar ratios of 10:1 and 100:1. Forty-eight hours posttransfection, the culture medium was replaced by fresh medium containing 15 µg/ml puromycin. Then, the cells were subcultured weekly in puromycin-containing medium until stably transformed polyclonal populations were established (approximately 4 weeks). Stably transformed cell lines were maintained in ESF 921 containing 50 µg/ml gentamicin (Invitrogen) and 15 µg/ml puromycin (Applichem). For suspension cultures (typically, a 100-ml volume in spinner flasks), the medium was also supplemented with Pluronic F-68 (Sigma) to a final concentration of 0.2%. Cell culture supernatants from each population were compared for inhibitory activity using the enzymatic (papain) assay described below, and the most active population (cotransfected at molar ratios of 100:1) was further amplified in large-scale stationary and suspension cultures for protein purification.

Purification of recombinant cystatin 1. Recombinant cystatin 1 was purified by affinity chromatography using a modification of previously described protocols (5, 14). Briefly, cell culture supernatants (from cultures grown typically for 7 to 10 days to a cell density of 2×10^6 cells/ml), were concentrated approximately 10 fold by ultrafiltration in a Centricon Plus ultrafiltration apparatus (Millipore Biomax-5; molecular weight cutoff, 5,000). Trypan blue staining of cells indicated that 95% of cells were viable upon harvesting. After desalting with a PD10 column (AP Biotech) in binding buffer (50 mM sodium phosphate, 0.1% Brij 35, 1 mM EDTA, pH 6.5), the protein fraction was mixed with 2 ml immobilized carboxymethylated papain (CM-papain; Calbiochem) preequilibrated with binding buffer and incubated overnight at room temperature. The gel matrix was poured into a column and washed with the same buffer until A_{280} was 0 (typically, 5 to 10 bed volumes). Bound material was eluted with elution buffer (50 mM K₂HPO₄/KOH, 0.1% Brij 35, 1 mM EDTA, pH 11.5) in 1-ml fractions. To each fraction, 0.2 ml of 1 M Tris HCl, pH 7.3, and 0.3 ml of 80% glycerol were added; samples were stored at –20°C. Protein samples from all purification steps were analyzed on 18% SDS-polyacrylamide gels stained either with Coomassie Blue or with silver. The purity and homogeneity of the purified recombinant cystatin 1 were assessed by N-terminal peptide sequencing using a Procise sequencer (Applied Biosystems, France).

Inhibition of cysteine proteases by cell culture supernatants and recombinant cystatin 1. E-64 was obtained from Sigma-Aldrich (St. Quentin le Fallavier, France), Z-Phe-Arg-AMC (7-amino-4-methyl-coumarin) and dithiothreitol (DTT) from Bachem (Weil am Rhein, Germany), papain (EC 3.4.22.2) from Boehringer (Roche Molecular Biochemicals, Mannheim, Germany), human cathepsin B (EC 3.4.22.1) and human cathepsin L (EC 3.4.22.15) from Calbiochem (VWR International, Pessac, France), and *Sarcophaga peregrina* 29-kDa cathepsin B (39). Cathepsins, papain, and the 29-kDa *Sarcophaga* cathepsin B were activated in 0.1 M phosphate buffer, pH 6.0, containing 2 mM DTT and 1 mM EDTA for 5 min at 37°C prior to kinetic measurements (spectrofluorimeter Kontron SFM 25), and their active sites were titrated with E-64 (6), using

Z-Phe-Arg-AMC as a substrate (excitation wavelength, 350 nm; emission wavelength, 460 nm).

Papain (1 nM), cathepsin B (1.8 nM), or cathepsin L (0.6 nM) and the 29-kDa *Sarcophaga* cathepsin B (approximately 1 nM) were incubated in the presence of increasing amounts of recombinant cystatin 1 (0 to 100 nM) for 30 min at 30°C in 50 μ l of assay buffer before Z-Phe-Arg-AMC (3.3 μ M) was added (to a final volume of 300 μ l), and residual amidolytic activity was measured by monitoring the release of the 7-amino 4-methylcoumarin group. The same protocol was repeated with human tissue kallikrein (Sigma-Aldrich), bovine trypsin and chymotrypsin (Roche Molecular Biochemicals), and pepsin (Sigma-Aldrich) (final concentration, 10 nM) using experimental conditions reported previously (44).

For activity assays of cell culture supernatants, papain (10 nM) was incubated for 10 min at room temperature in 450 μ l of stock assay buffer (0.1 M Na phosphate buffer, pH 6.0, and 2 mM DTT) with 50 μ l of cell culture supernatants (10% [vol/vol]) before the addition of Z-Phe-Arg-AMC (Bachem Biochimie, SARL, France) to a final concentration of 100 μ M. The relative activity of each supernatant was determined by comparison with a standard curve (without cell culture medium) or with curves obtained from control cell culture supernatants.

Nucleotide accession number. Cystatin nucleotide and protein sequences have been deposited in the EMBL database, under accession no. AJ632321.

RESULTS

Isolation and mapping of CcBV cystatin genes. To isolate viral genes expressed in parasitized host caterpillars, a CcBV genomic library was constructed. Clones obtained from this library were subsequently screened by reverse Northern analysis, using as a probe radiolabeled cDNA derived from poly(A)⁺ mRNA of *M. sexta* larvae 24 h postparasitization. Upon being sequenced, one of the hybridizing clones giving the strongest signals, E6 (a 6-kb-long EcoRI insert), was found to encompass an ORF encoding a putative protein with homology to the cystatin superfamily. Subsequent screening of a cDNA library generated from parasitized *M. sexta* larvae, using the E6 clone as probe, resulted in the isolation of two cDNA clones designated cystatin 1 and cystatin 2. The sequence of cystatin 1 was identical to that contained in the E6 genomic fragment, while that of cystatin 2 was similar (Fig. 1A).

We undertook the mapping of the cystatin genes in the viral genome. Field inversion gel electrophoresis of the viral genome, followed by Southern blot analysis, revealed that only one segment of the CcBV genome, a DNA circle of an estimated linear size of 19 kb (Fig. 2A), contained sequences related to the cloned cystatin genes. Subsequent sequencing of the entire viral genome (Genoscope-French National Sequencing Center, Evry, France) confirmed that the two cloned cystatin genes were indeed located on a single circle and further revealed the existence of a third cystatin gene (cystatin 3) on the same circle (circle 19; 18,768 bp) (Fig. 2C) (22).

The map of the cystatin gene-containing circle was confirmed experimentally using Southern blots of viral DNA digested with different restriction enzymes and cystatin 1 and cystatin 2 probes (Fig. 2B and C). Strong hybridization signals were obtained with probes of the corresponding genes; however, cross-hybridization signals of weaker intensities were observed between cystatin 1 and cystatin 2 (Fig. 2B). All the bands of the expected size were obtained with varying intensities, depending on the probe used. In addition a 2.3-kb band was detected with viral DNA digested by BamHI, corresponding to a partial digestion obtained by absence of digestion of a particular BamHI site (position 914) (Fig. 2C). No polymorphism was identified at this site during genome sequencing, and only this site showed incomplete digestion. This suggests it

may not be easily accessible to the enzyme, due to a secondary structure of the viral DNA in this part of the circle.

Sequence characterization of CcBV cystatin genes and products. Analysis of the genomic and cDNA sequences of cystatin 1 and cystatin 2 genes and the genomic sequence of the cystatin 3 gene revealed that no introns were present in these genes. Furthermore, the analysis showed that all three genes were very similar: the cystatin 1 gene exhibited 89% similarity to the cystatin 2 and cystatin 3 genes, and the cystatin 2 and 3 genes showed 94% identity (blastn program, National Center for Biotechnology Information) (Fig. 1A).

The cystatin 1, 2, and 3 genes encode proteins of 135, 134, and 134 amino acids (theoretical molecular masses of 15.42, 15.45, and 15.47 kDa), respectively, which are related to members of the cystatin superfamily of proteins. They harbor hydrophobic N-terminal domains, putatively signal peptides, with potential cleavage sites at residues 22 (cystatin 1) and 24 (cystatins 2 and 3) (Fig. 1B) (49). Cystatin 1 possesses a putative tyrosine phosphorylation site at position 121 to 127, and cystatins 3 and 2 contain one and two potential Asn-linked glycosylation sites, respectively (Prosite, ExPASy; <http://www.expasy.org/prosite/>), in accord with published reports suggesting that at least some cystatins are phosphorylated (40) and glycosylated (20).

Most notable, however, is the existing conservation of the presumed inhibitory domains of the proteins, which include a N-terminal glycine residue at position 28 (Fig. 1B) corresponding to amino acid 6 of the mature protein (see below) and glycine 9 of the mature chicken cystatin protein (Fig. 3B), the QX(V)XG consensus motif beginning at position 72, proline 118 (for cystatin 1 only), and a tryptophan residue at position 119 at the C terminus (Fig. 1B and 3B). X-ray crystallographic analysis of the chicken cystatin suggests that these domains form a wedge that can interact with the active site of papain (13). Cystatin 1 showed the strongest conservation in the consensus QVVAG pentapeptide, whereas cystatin 2 and cystatin 3 harbored a QVLYG sequence (Fig. 1B and 3B). Altogether, the three potential cystatins have all the characteristic features of functional cystatins.

Related cystatin proteins. The cystatin superfamily consists of three families, the stefins (or type 1), the cystatins (type 2), and the kininogens (type 3) (2). The CcBV cystatins show most resemblance to the type 2 cystatins, particularly type 2 chicken and quail cystatins, human cystatins C and D, mouse cystatin 10, and filarial parasite *Brugia malayi* cystatin Cpi-1 (Fig. 3). Strong similarities were also observed with the type 3 cystatin domain of bovine kininogen (Fig. 3).

Type 2 cystatins are generally secreted proteins consisting of a single domain with four cysteine residues potentially forming two disulfide bonds (2). Well-described type 2 cystatins include chicken cystatin and human C, D, S, SA, and SN cystatins (2), which contain two conserved disulfide bridges at their C-terminal domains (2, 56). In cystatin 1, however, the first disulfide bridge does not have the same spacing as the avian or mammalian type 2 cystatins (Fig. 3B), while cystatins 2 and 3 contain an extra cysteine between the disulfide bridges (Fig. 1B and 3B).

Because the CcBV genome is integrated in the parasitoid wasp genome, we compared the sequences of CcBV cystatins to known insect cystatins (Fig. 3A), in an effort to deduce

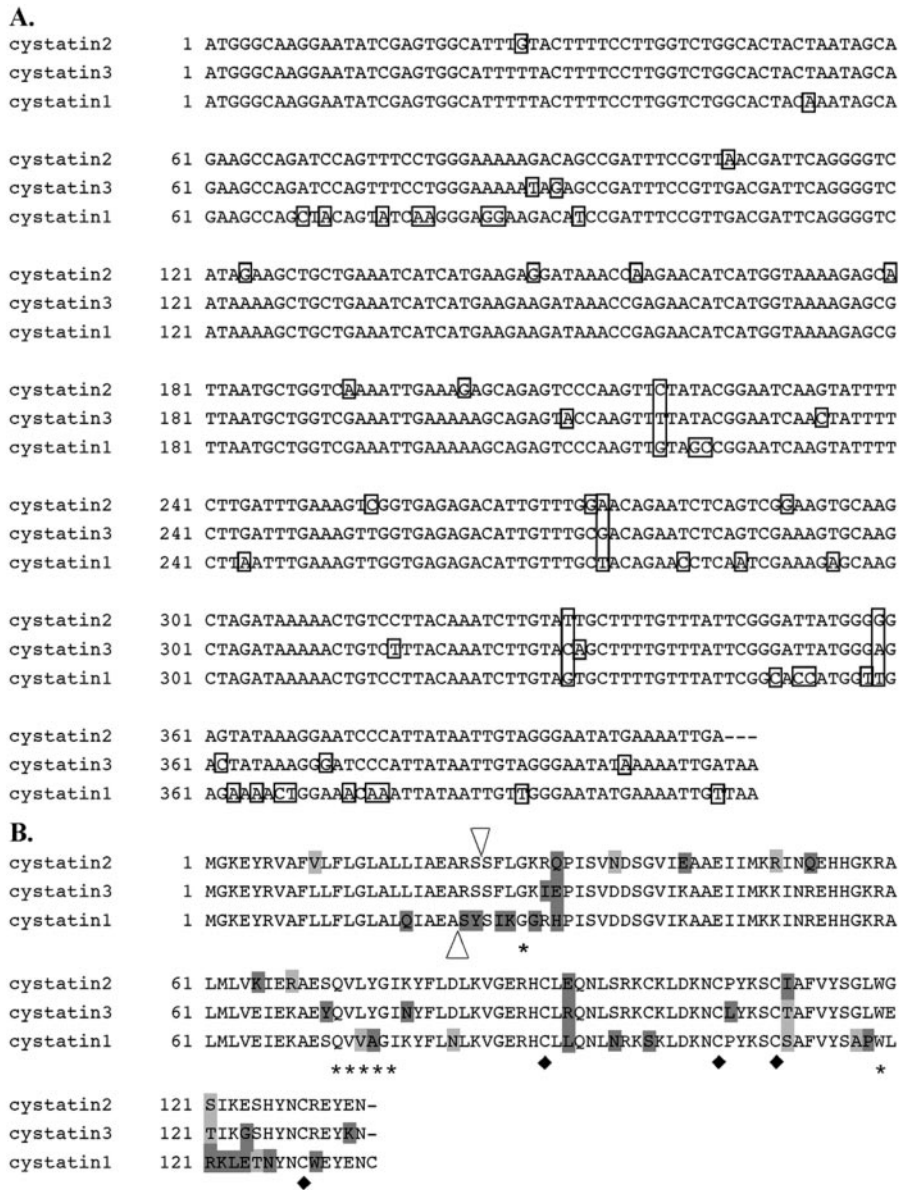


FIG. 1. Nucleotide and amino acid sequence alignment of the CcBV cystatins. (A) Nucleotide sequence alignment of the three CcBV cystatin ORFs. Differences in nucleotide sequences are boxed. (B) Amino acid sequence alignment of the three CcBV preprotein cystatins. The potential cleavage site for cystatin 2 and cystatin 3 (same site) and cystatin 1 are indicated by white triangles. The three active sites which are the hallmarks of cystatins are indicated by stars: G (residue 28), Qx(V)xG (starting residue 72), and W (residue 119). The four cysteine residues potentially forming two disulfide bonds are indicated by black diamonds. Differing amino acids are boxed (same family, light grey boxing; different family, dark grey boxing).

whether the viral cystatin genes may have been acquired from the wasp genome. Comparisons with four *Drosophila* genes encoding putative cystatins CG15369 (Q9W370-DROME), CG8066 (Q9VF13-DROME), CG31313PA (Q85ZNI-DROME) (<http://flybase.bio.indiana.edu/>), and Cys (CYTL-DROME) (19) and genes encoding flesh fly sarcocystatins (*Sarcophaga peregrina*) (55) and *Sarcophaga crassipalpis* (28) have revealed the existence of similarities between cystatin 1 and sarcocystatin A of *S. peregrina* (Fig. 3A). Comparison with the predicted amino acid sequence for a cystatin superfamily member identified in the mosquito genome database (Ano-

base) revealed 25% identity and 44% similarity with cystatin 1 and similar values with cystatins 2 and 3 (Fig. 3A). Furthermore, a sequence identified in the honeybee genome, which encodes a putative cysteine protease containing a type 2 cystatin domain at its N terminus, showed 29% identity and 47% similarity to cystatin 1 and similar values with cystatins 2 and 3. These data strongly suggest that the CcBV cystatins may, in fact, have a cellular origin.

The phylogenetic relationship between bracovirus, vertebrate, and invertebrate cystatins was analyzed by the methods of distance and parsimony. Not surprisingly, bracovirus cystatins

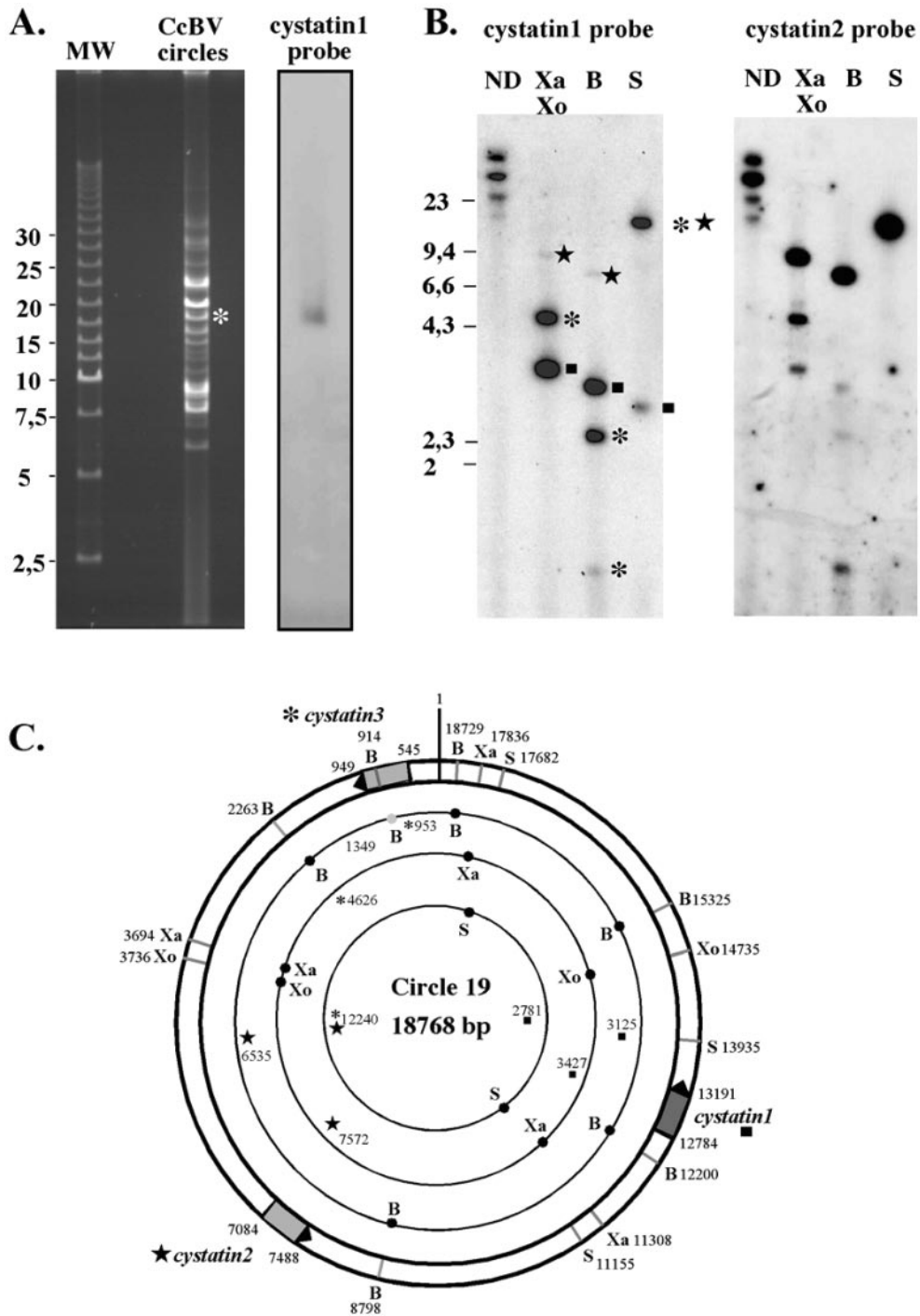


FIG. 2. Mapping of *Cotesia congregata* bracovirus cystatins in the viral genome. (A) Field inversion gel electrophoresis of 250 ng of viral DNA, followed by hybridization using cystatin 1 cDNA as a probe. The unique hybridization signal observed corresponds to the circle (indicated by a white asterisk) of the estimated linear size (19 kb). The 2.5-kb ladder from Bio-Rad was used as linear molecular weight marker in this experiment. (B) Southern blot analysis of viral DNA (200 ng per lane), nondigested (ND) or after restriction digestion with XbaI-XhoI (Xa, Xo), BamHI (B), or SacI (S), using either cystatin 1 cDNA or cystatin 2 cDNA as a probe. Hybridizing fragments corresponding to cystatin 1 are indicated by a black square. Hybridizing fragments corresponding to cystatin 2 are indicated by a black asterisk. Hybridizing fragments corresponding to cystatin 3 are indicated by a black star. (C) Restriction map of CcBV circle 19, harboring the three cystatin genes. Lengths of expected fragments after restriction digestion are indicated. Hybridizing fragments are highlighted with corresponding symbols (box, star, or asterisk). Note the presence of a BamHI site (grey circle) in cystatin 3, which gives rise to a partial digest.

A.

	CcBV Cys1	CcBV Cys2	CcBV Cys3	Sp SarcocysA	Amel	Agam	Gg Cys	Bm Cpi-1	Hs CysC	Mm Cys10	Bt Kininogen
CcBVCys1	100	76/85	78/83	30/46	29/47	25/44	28/51	28/50	28/48	26/41	29/44
CcBVCys2		100	77/84	NS	27/48	23/46	28/50	26/48	23/42	25/44	26/46
CcBVCys3			100	NS	27/46	26/50	28/50	26/46	25/52	27/46	27/46

B.

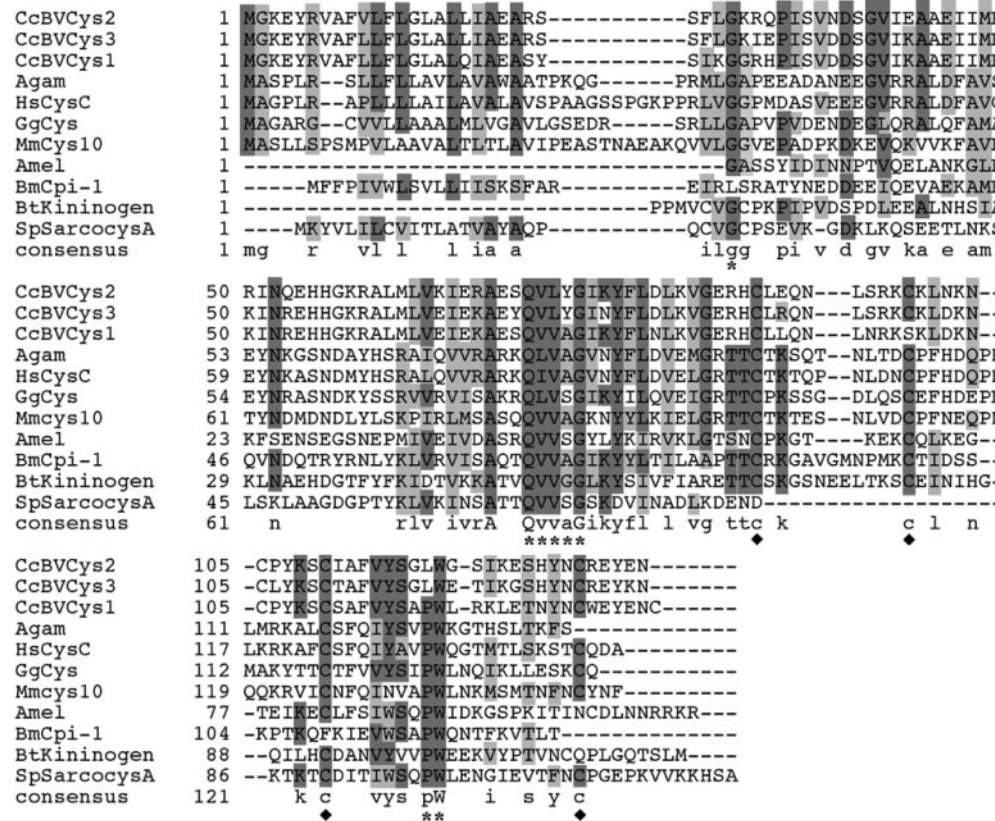


FIG. 3. Pairwise BLAST and multiple sequence alignment of CcBV cystatins with insect and noninsect cystatins. (A) Pairwise BLAST of CcBV cystatins with insect and noninsect cystatins. Numbers indicate percentage identity/percentage similarity. Preprotein sequences were compared. (B) Multiple sequence alignment of CcBV cystatins with related cystatins using the Clustalw program (<http://clustalw.genome.jp/>) combined with the BoxShade Server (http://www.ch.embnet.org/software/BOX_form.html). Preprotein sequences were compared in this alignment. The numbering refers to CcBV Cystatins labeled with the number one have the first amino acid of the signal peptides. The three conserved active sites of cystatins are indicated by stars beneath the consensus sequence: G residue, Qx(V)xG motif and (P)W residues. The cysteine residues in conserved positions are indicated by black diamonds. Agam, *Anopheles gambiae* (mosquito, Diptera; GenBank accession number EST AJ284933); Amel, *Apis mellifera* (honeybee, Hymenoptera; Honeybee Genome Project accession number Amel_1.contig2738); Bm, *Brugia malayi* (filarial nematode; GenBank accession number AAC47623); Bt, *Bos taurus* (bovine; GenBank accession number P01045); Gg, *Gallus gallus* (chicken; GenBank accession number NP_990831); Hs, *Homo sapiens* (GenBank accession number NP_000090); Mm, *Mus musculus* (mouse; GenBank accession number NP_067380); Sp, *Sarcophaga peregrina* (flesh fly, Diptera; SwissProt accession number P31727).

constitute a clade indicating that the corresponding genes originated from a common ancestor (unambiguously, a cellular cystatin gene) that went through two rounds of duplications. The wasp is the likely source of the ancestral cystatin gene, but the high rate of divergence of bracovirus genes involved in host-parasite interactions (51) would prevent the isolation of the ancestor gene in *C. congregata*.

Expression of bracovirus cystatins in the parasitized host. Detecting the expression of different cystatin genes during

parasitism would constitute a first indication of their involvement in bracovirus-induced alteration of host physiology. We already knew by the reverse Northern analysis that at least one of the genes was highly expressed. We therefore determined the expression of all three cystatin genes at different time points postparasitism and in different tissues by Northern blot analysis with cystatin 1 cDNA as a probe.

A time course experiment using RNA extracted from parasitized host fat body showed that the cystatin genes are ex-

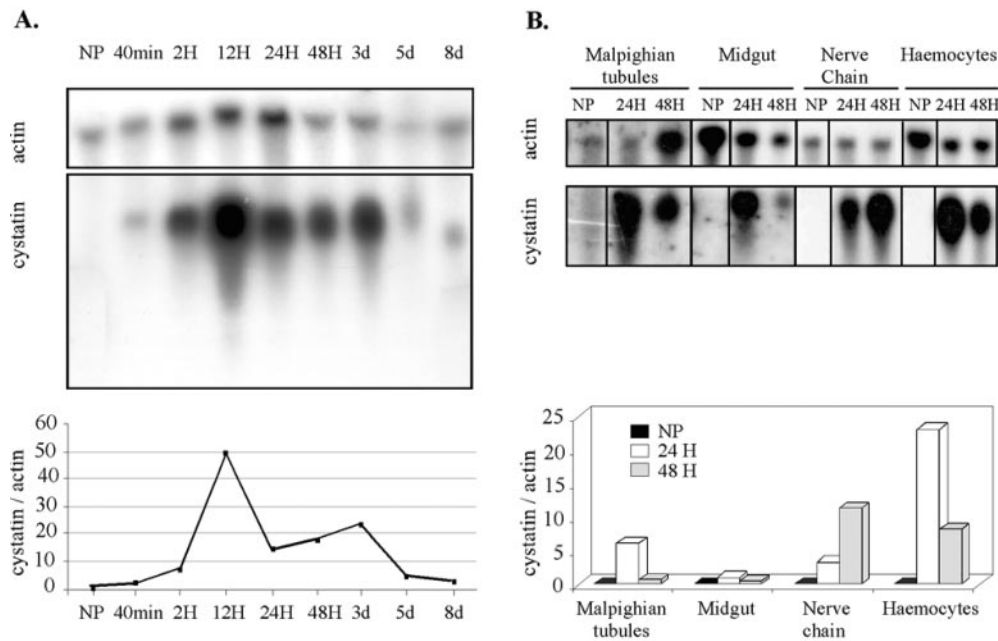


FIG. 4. Expression profile of CcBV cystatin genes in parasitized *Manduca sexta* by Northern blot analysis (A) Time course experiment using RNA extracted from nonparasitized (NP) or parasitized *Manduca sexta* fat body at different time points postparasitization as indicated. (B) Tissue-specific expression using RNA extracted from nonparasitized (NP) or parasitized *Manduca sexta* at different time points postparasitization as indicated. A total of 20 μ g of RNA was loaded in each lane. Cystatin 1 cDNA was used as a probe. As a control, blots were hybridized with *Manduca sexta* actin. Levels of cystatin expression are indicated relative to that of the control actin. The values correspond to the ratios obtained after Instant Imager scanning of the Northern blots presented.

pressed throughout parasitism (Fig. 4A). Expression could be detected as early as 40 min after wasp oviposition and reached a peak at 12 h postparasitism in the fat body. In the experiment shown, cystatin gene expression was estimated to reach 50 times the level of actin at this particular time point. After 3 days, a severe drop in expression was observed (Fig. 4A). No hybridization signals were obtained in nonparasitized host tissues.

Analysis of the expression of viral cystatins in other tissues of parasitized host showed that cystatin genes are also abundantly expressed in hemocytes at 24 h postparasitism (Fig. 4B). Strong expression was also observed in the nerve cord and brain at 48 h, as well as in Malpighian tubules at 24 h postoviposition. No cystatin RNAs could be detected in male wasps, and only a barely detectable signal was observed in female wasps (data not shown), suggesting that the cystatin genes are highly expressed in parasitized caterpillars only.

Although equal amounts of RNA were loaded in each lane, levels of actin transcripts were found to vary during the course of parasitism and between the different tissues tested. Variability in host control transcript levels has already been observed in other host-parasitoid systems (35). One explanation could be that the ratios of host RNA versus viral RNA vary during parasitism or that one of the effects of the virus is on host transcription. Despite the difficulty in finding a nonvariable control, results presented here have a qualitative value and give the general trend of cystatin gene expression.

To determine whether all three cystatin genes are expressed in the host during parasitism, RT-PCR was performed on mRNA obtained from a 24-h parasitized caterpillar fat body,

using primer couples specific for each gene. The results demonstrated that all three viral cystatins are expressed during parasitism (data not shown).

Recombinant cystatin 1 expression and purification. To determine whether one of the viral cystatins could be a functional cysteine protease inhibitor, expression of recombinant cystatin 1 was achieved using a lepidopteran cell-based expression system. Transient expression in insect cells was performed; 3 days posttransfection, cell culture supernatants were assayed for inhibition of papain. The supernatants exhibited significant inhibition of papain compared to nontransfected cell supernatants, suggesting that the lepidopteran cell system enables both expression and secretion of an active form of cystatin 1 into the culture medium.

High-level expression of cystatin 1 protein was achieved by generating two stably transformed cell lines, HI5-C10 and HI5-C100. Supernatants from the two cell lines were assayed for inhibition of papain, and both lines exhibited significant inhibition of papain compared to nontransfected cell supernatants (Fig. 5). Line HI5-C100 was selected for further amplification to proceed with the purification of cystatin 1 protein.

The recombinant cystatin 1 present in the supernatants of HI5-C100 cell cultures was subjected to purification on the basis of its affinity for immobilized inactivated (carboxymethylated) papain. Flowthrough, wash, and eluate fractions were examined electrophoretically (Fig. 6) and also assayed for papain inhibitory activity. A single band at the expected molecular mass was observed in the eluate fractions (Fig. 6, lanes 6 and 7), and the same fractions (but not the flowthrough or wash fractions) were found to cause significant inhibition in

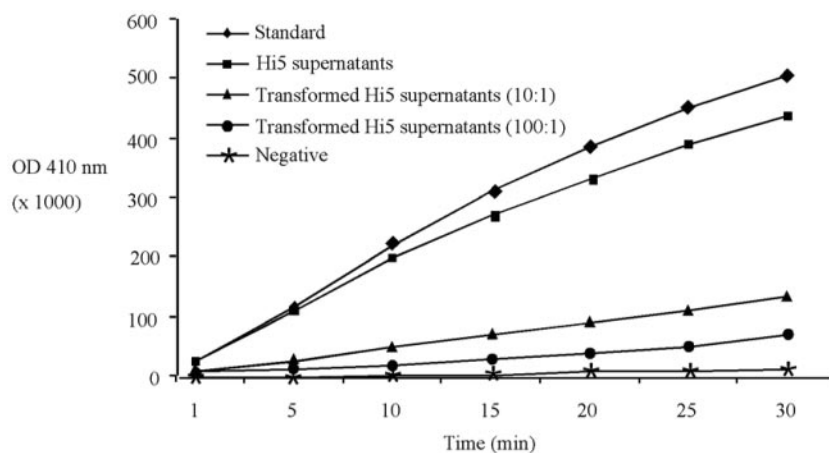


FIG. 5. Enzymatic assays for evaluation of stably transformed cell lines expressing recombinant cystatin 1 protein. The assays were performed using 10% (vol/vol) cell culture supernatant in each reaction mixture as described in Materials and Methods. The following conditions are shown: standard, standard curve without inhibition; transformed Hi5 supernatants (10:1 and 100:1), supernatants from the relevant stably transformed cell lines (HI5-C10 and HI5-C100) expressing cystatin 1 protein; Hi5 supernatant, control supernatant from nontransfected HighFive cell cultures of the same cell density as transfected Hi5 cells; negative, assay without papain to examine substrate self-hydrolysis. Note that addition of 10% control supernatant had a very minor impact on the assay kinetics, while supernatants from cystatin 1 expressing cultures inhibited papain very strongly. The effect of self-hydrolysis was negligible.

functional inhibition assays (see below). The N-terminal peptide sequence SYSIKGGRH confirmed that the single band observed in the eluate fractions corresponded to cystatin 1 and that the protein was cleaved after position 22 as predicted (Fig. 2B), yielding the first amino acid of the mature form.

Inhibitory activity of CcBV cystatin 1. Recombinant CcBV cystatin 1 was assayed for protease inhibitory activity with representative cysteine proteases of the C1 family (clan CA). Cystatin 1 exhibited a highly potent inhibitory activity towards papain, with approximately 80% inhibition being achieved at an equimolar ratio of enzyme to inhibitor (Fig. 7A). Human cathepsin B and L activities towards Z-Phe-Arg-AMC were also dramatically inhibited, but to a lesser extent than papain (Fig. 7B). On the other hand, the amidolytic activities of tissue

kallikrein hK1, pepsin, trypsin, and chymotrypsin were not affected by recombinant cystatin 1 (data not shown), confirming that native CcBV cystatin 1 is a specific cysteine proteinase inhibitor.

During the parasitoid-host interaction *in vivo*, cysteine proteases of the C1 family are very likely to be the targets of CcBV cystatin 1. We therefore wanted to determine if recombinant cystatin 1 could also inhibit a C1 cysteine protease of insect origin. Recombinant CcBV cystatin 1 was assayed for protease inhibitory activity with an available 29-kDa cysteine protease from *Sarcophaga peregrina* (39). This 29-kDa protease, which has been described as *Sarcophaga* cathepsin B (66), is secreted by pupal hemocytes and is involved in decomposition of larval fat body during metamorphosis (38). Cystatin 1 displayed potent inhibitory activity towards this insect cathepsin (Fig. 7B), indicating that C1 family cysteine proteases are potential targets of viral cystatins in *M. sexta*.

DISCUSSION

Cystatin superfamily members have been described for numerous organisms (2, 15, 53). Here, we report for the first time the isolation and characterization of three cystatin genes associated with a virus, CcBV. All three genes encode type 2 cystatin superfamily members, and all are expressed at a high level during *C. congregata* parasitism of its host *M. sexta*. Expression and purification of a recombinant form of CcBV cystatin 1 demonstrated that this viral cystatin is a functional cysteine protease inhibitor, with potent inhibitory activity towards papain, human cathepsins B and L, and *Sarcophaga peregrina* 29-kDa cathepsin B.

Scenario of viral cystatin gene acquisition and evolution. Braconid wasp species harboring polydnviruses have been shown to form a monophyletic group, the microgastroid complex, composed of seven subfamilies (Cheloninae, Dirrhopiinae, Mendesellinae, Khoikhoiinae, Cardiochilinae, Miracinae,

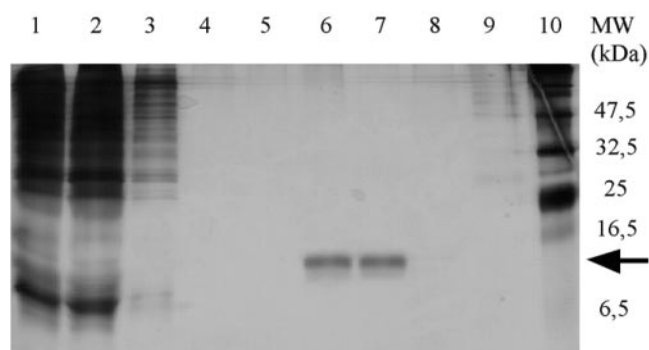


FIG. 6. Silver-stained SDS-polyacrylamide gel electrophoresis (18%) gel of 20- μ l samples from several steps of the cystatin 1 protein purification procedure. Lanes: 1, concentrated, desalted cell culture supernatant before application to CM-papain matrix; 2, postbinding flowthrough fraction; 3 and 4, wash fractions (5 bed volumes each); 5 to 8, eluate fractions (1 bed volume each); 9, CM-papain matrix beads after elution; 10, molecular mass standard. The arrow points to the prominent protein band that migrates at the expected molecular weight for cystatin 1 (see text).

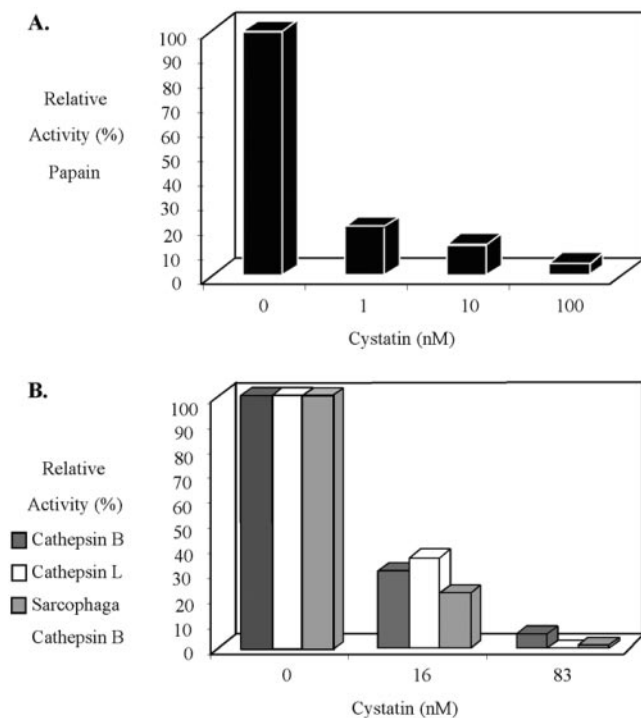


FIG. 7. Cysteine protease inhibitory activity of CcBV cystatin 1. Various concentrations of cystatin 1 were incubated with papain (1 nM) (A) or human cathepsin B (1.8 nM), cathepsin L (0.6 nM), or *Sarcophaga* cathepsin B (approximately 1 nM) (B). The relative activity of each cysteine protease was determined in presence of the recombinant cystatin 1. Incubation of each protease with the substrate and without addition of cystatin 1 corresponded to 100% activity of the enzymes.

and Microgastrinae) comprising 17,500 species (76). One of the current hypotheses concerning the origin of these wasp-bracovirus associations is that they originated from the integration of an ancestral bracovirus in the chromosome of an ancestor wasp that lived approximately 70 million years ago (73). Under this hypothesis, all contemporary bracoviruses should have common genes inherited from the ancestral virus. Indeed, conserved genes have been identified both in closely related *Cotesia* wasp species (43, 75) and even in two bracoviruses, *Toxoneuron nigriceps* bracovirus and CcBV of two different braconid subfamilies, Cardiochilinae and Microgastrinae, respectively (51). To date, cystatin genes have been identified in two polydnviruses of the Microgastrinae subfamily, namely, CcBV and *Glyptapanteles indiensis* bracovirus (D. Gundersen-Rindal, personal communication), but we already have evidence that cystatins are not present in the *Toxoneuron nigriceps* bracovirus genome of the Cardiochilinae subfamily (F. Pennacchio, personal communication). Furthermore, CcBV cystatins are much less divergent than other CcBV protein families (51). Altogether, these data suggest that cystatin genes were acquired by bracoviruses relatively recently (at least since Microgastrinae and Cardiochilinae diversification, approximately 50 million years ago) (76) and were subsequently duplicated in the viral genome.

The fact that the CcBV cystatins show significant homology to insect cystatins and that they have the conserved structure of

cellular type 2 cystatins suggests that CcBV acquired these gene(s) from the wasp genome. Moreover, because cystatin genes may be present only in a subset of bracovirus genomes, it is unlikely that these gene(s) were present in the genome of the ancestral bracovirus. Lastly, because many PDV genes described contain intronic sequences (68) and because all reported cellular cystatin genes also contain introns of characteristic size and location (15), the absence of introns from CcBV cystatin genes suggests that the virus acquired the ancestral gene(s) via integration of cDNA. A similar suggestion has been made for the case of the vinnexin genes of the polydnvirus of the parasitoid wasp *Camponotus sonorensis* (68). Interestingly, human long interspersed element retrotransposons have been shown to be able to mobilize transcribed DNA sequences not necessarily associated with a long interspersed element. This results in the generation of processed (i.e., intronless) pseudogenes which can be expressed and fulfill new physiological functions (21). Accordingly, the most plausible scenario is that an ancestral CcBV cystatin gene was acquired from the host wasp genome by integration of cDNA and further duplicated during evolution. Identification of cystatin genes in other bracoviruses from the Microgastrinae subfamily could be useful in pinpointing the time of acquisition of these genes by the integrated form of the virus.

Multigene families, of frequent occurrence in polydnviruses (37, 72), are thought to arise from gene duplication events (11). It is likely that a high selection pressure is exerted on PDV genes, resulting in efficient evasion of host defense systems or expansion of the wasp's host range. Closely related genes may provide redundancy in function or allow synergistic effects on different host targets. Alternatively, different gene variants may be required for wasp development in different host species. The presence of multiple genes may also enable abundant expression of important functional genes in the parasitized host, particularly in the absence of viral replication in the host caterpillar.

Potential roles of viral cystatins in the parasitized host. Several lines of evidence suggest that cystatins are likely to play important roles in alteration of host caterpillar physiology. Recombinant CcBV cystatin 1 is a functional and specific cysteine protease inhibitor. In addition, cystatin genes are rapidly and highly expressed in the host caterpillar after wasp oviposition, and expression is maintained throughout parasitism. This early and prolonged expression could be indicative of a role of cystatins in the early steps of host physiological disruption, as well as in the maintenance of this perturbed state. Relevant to this is the fact that injection of purified virus in *M. sexta* larvae reproduces many of the parasitism-induced effects, including partial arrest of host larval development and induction of host immunosuppression (8, 10). Furthermore, cellular cystatins have been reported to act as potential regulators of development and as immunomodulatory proteins (see below). Accordingly, we now need to understand the precise role of viral cystatins in the modulation of the host physiology.

Cystatins and programmed cell death. Some viruses are known to harbor inhibitor of apoptosis (IAP) genes, which act by suppressing host defense mechanisms that would otherwise eliminate virus-infected cells by apoptosis (16, 33). Induction of apoptosis in hemocytes (63) and hematopoietic organs (67) has been described for two bracovirus-host parasitoid interac-

tions. A gene that induces apoptosis-like programmed cell death (PCD) in insect cells has been described for *T. nigricipes* bracovirus (41). After CcBV injection, *Manduca sexta* hemocytes are blebbed and collapsed, indicating they could also be undergoing apoptosis (42). Could CcBV cystatins represent a new class of IAPs? So far, the viral IAPs described are caspase inhibitory proteins (16, 33). Representative members of all three families of cystatins were tested for inhibition of caspases 3, 6, 7, and 8; no inhibition was observed even at vast excess of cystatins (61; G. Salvesen and M. Abrahamson, personal communications). Caspases are therefore not the direct targets of CcBV cystatins.

Interestingly, the lysosomal cysteine proteases cathepsin B and L can trigger both caspase-independent and caspase-dependent programmed cell death (34). In most models of lysosome-dependent death, cathepsins translocate from lysosomes to the cytosol or nucleus before the appearance of morphological changes indicative of PCD (34). Therefore, cystatins could be involved in inhibition of hemocyte PCD via the inhibition of lepidopteran cathepsins. Given the most probable extracellular localization of CcBV cystatins, however, it is unlikely that cystatins could truly be involved in this pathway.

Programmed destruction of tissues that are no longer needed or that need to be renewed (i.e., during moulting or metamorphosis) is an essential developmental process in which cysteine proteases have been reported to be involved (32, 39, 46, 60, 66, 78). In the flesh fly, *S. peregrina*, successful development relies on the production and secretion of family C1 cysteine proteases (32, 38, 39); an endogenous cystatin has been described, sarcocystatin A, which is believed to act as a modulator of cysteine protease activity during development, thereby protecting newly developing larval or adult tissues (55, 64, 65). In *M. sexta*, little is known about the proteolytic enzymes involved in larva-pupa molts. Although recombinant CcBV cystatin 1 inhibits *Sarcophaga* cathepsin B involved in fat body degradation, it is not clear whether this could be the true biological function of viral cystatins. Indeed, host caterpillar developmental arrest during parasitism is attributed in general to endocrine deregulation, which affects early upstream events in the developmental process (8). The parasitoid wasp itself has been shown to release juvenile hormone (17) and to manipulate ecdysteroid titers in vivo (27); CcBV is also suspected to be involved in host endocrine deregulation, although the direct action of CcBV on these targets has yet to be demonstrated (8).

Cystatins and immunomodulation. Cystatins have also been described as immunomodulatory molecules (69, 71). In filarial nematodes, for example, cystatins appear to account for a major portion of the immunosuppressive activity of secreted filarial proteins and are therefore considered major pathogenicity factors of filariae (30, 47, 59). Filarial cystatins induce tumor necrosis factor alpha, followed by a strong production of the immunosuppressively acting interleukin 10 cytokine, and interfere with specific and nonspecific proliferation of host T cells (59, 71). It would, therefore, be interesting to investigate whether CcBV cystatins could be involved in the modulation of immune cell proliferation in the host.

Interestingly, a cysteine protease purified from *S. peregrina* larval hemocytes has been shown to be involved in the selective degradation of non-self proteins (54). This cysteine protease of

the papain family, which is released into the hemolymph after injection of foreign bodies or wounding, could be involved in the elimination of foreign substances and/or wound healing (54). This proteinase has been shown to be conserved in a wide variety of insects (26) including *M. sexta* (E. Huguet, unpublished results). If released in *M. sexta* hemolymph in response to parasitism, this cysteine protease might constitute the target of viral cystatins that may act to inhibit degradation of viral and/or wasp proteins in the lepidopteran host. The early expression pattern of cystatins suggests that they could be involved in inhibiting immediate host immune responses. Furthermore, the fact that recombinant cystatin 1 can inhibit a similar cysteine protease from *Sarcophaga* in vitro and that cystatins and the potential cysteine protease(s) are likely to have an extracellular localization also argues in favor of this hypothesis and could constitute a starting point for future investigations of cystatin targets in vivo.

In conclusion, in addition to the recently characterized CcBV protein tyrosine phosphatases (51), CcBV cystatins represent some of the few CcBV proteins for which a functional activity (i.e., specific protease inhibitor) has been established. Our attention will now be directed towards the identification of the targets of the viral cystatins in the host caterpillar. The characterization of such targets will help us to deduce the host physiological processes affected by viral cystatins and may help us to establish parallels between the different virulence strategies used by very different types of parasites in the host takeover process.

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